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THE INFLUENCE OF MERISTEMATIC TISSUE AND INJURIES ON THE TRANSPORT OF TOBACCO MOSAIC VIRUS IN NICOTIANA TABACUM L. CULTIVAR. SAMSUN

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1. INTRODUCTION

It has been stated by several authors that the tops of the stem, the axillary buds, and the roottips of a plant infected with virus are of influence on the rate and direction of virus-transportation. There has been, however, no agreement about the effect of these plant-parts. Some authors suggest an accelerating, others an inhibiting effect of the stemtop on the rate of transportation. This discrepancy can be explained by the difference in methods used by these authors, by the difference in hostplants and virus-material and by the limited number of experiments which were carried out. Moreover these experiments were founded on one principle, namely of removal of the parts, and the influence of the stemtops or the axillary buds was studied by comparing the rate of virus-transportation in normal plants with that in plants from which these parts had been removed (Grainger, 1933; Bennett, 1940; Zech, 1952).

This method has two distinct disadvantages. 1) Since removal of these plant-parts may eliminate more than one kind of tissue, it is impossible to determine which tissue had influenced the rate of virus-transport. The meristematic tissues such as the vegetation-point or the leaf-primordia, the young leaves, or parts of the stem may play a rôle. 2) Injuries are made during removal of these parts which may influence the metabolism of the plant and consequently the rate of virus-transportation. Neither the influence of wounding nor the influence of meristematic tissue on the rate of virus-transportation in a plant has ever been examined.

The purpose of our experiments was to verify the results reported in literature and to determine the effect of the influence of meristematic tissue on the rate and possibly the direction of virus-transport.

Plants of *Nicotiana tabacum* L. cultivar. Samsun were used for the experiments. The tobacco mosaic virus (*Nicotiana* virus 1 Smith) was chosen and will be indicated in this paper as TMV.

In spite of the difficulties mentioned above, a method of removal of meristematic parts and axillary buds was applied here. However, only very small stemtips [1]) of known morphology were removed and the influence of the wounding caused by this removal was studied.

When it became evident that wounding influenced the rate of virus-transportation positively, another method was sought by which the activity of meristematic tissue could be eliminated without wounding. Temporal inactivation of meristematic tissue was achieved by powdering the stemtip and the axillary buds with 2, 3, 5, 6-tetra-chloro-nitrobenzene, Fusarex.

It was also desirable to study the influence of meristematic tissue in an active condition on the rate of virus-transportation. Transplantation of such a tissue into a plant cannot be performed without wounding, and the influences of other meristematic tissue or tops present

[1]) In this publication a stemtip is defined as the extreme point of the stem with a length of maximal 2 mm, mainly consisting of meristematic tissue. The term stemtop is used when it concerns parts of more than 2 mm.

in a whole plant are not excluded. To comply with all conditions a homogeneous tissue, callus-tissue of tobacco, cultivated in vitro, was chosen for a third group of experiments. It proved to be possible to introduce a prepared meristem of a stemtip of 0.2 mm or a young stemtip of 2 mm on the callus culture without wounding the callus and without loss of activity of the meristem. In this way the influence of either a meristem or a stemtip could be determined, excluding the other meristematic influences.

To determine the rate of virus-transportation in a plant it is necessary to detect which tissue is first reached by the virus-material after inoculation. If the top of the stem and the axillary buds or a part of these organs have an attractive action, the virus-material leaving an inoculated leaf will first pass via the stem to these extremities of the plant and then to the other leaves. The virus-transportation through the stem takes place at such a high rate that some parts of the stem may remain virus-free in spite of the fact that virus-material passed by (Samuel, 1934; Kunkel, 1939; Beemster, 1958). For this reason the stem is not suitable as an indicator of the rate of virus-transportation. Therefore the leaves were chosen to determine which places in a plant are first attacked by the virus-material. Parts of the leaves may also be passed by without retaining any infectious material. However, in the leaf as a whole, virus-material will be present.

Just after arrival in uninoculated leaves the amount of virus is too small to be determined with the existing methods and, moreover, it is possible that the virus-material is not yet infectious at the moment of arrival.

Presence of very small amounts of non-infectious virus-material in a leaf is demonstrable only after multiplication during a period of incubation. For this purpose the leaves were removed from inoculated plants. The leaves were placed with their bases into water and incubated for three days. Under this condition virus-material, already present in the leaves at the moment of cutting, was allowed to multiply during three days, after which infectious virus-material became demonstrable with the local lesion test on *Nicotiana glutinosa*-leaves. The presence of virus can be demonstrated also serologically but the local lesion test appeared to be the more sensitive of the two.

With this method, however, it is impossible to prove whether the meristematic tissue of the stemtip, the axillary buds or the leaf itself influences the virus-transportation. But if a stemtip or the axillary buds attract the virus-material, removal or inactivation of these plant-parts must change the rate of virus-transportation and the moment of appearance of virus-material in the adjacent leaves.

2. LITERATURE

2.1. Virus-transportation in plants

It is known that after inoculation of a leaf of a plant with a virus causing systemic infection, there is an initial period of virus-multiplication in local sites of the inoculated leaf. This period is followed by a

comparatively sudden appearance of virus in all parts of the stem, the roots and the topmost leaves. Later the virus spreads to the lower leaves (Holmes, 1930; Samuel, 1934).

The way in which viruses are transported through a plant has already been described by many authors. A review of the literature is given by Beemster (1958).

Several conflicting data concerning the way of virus-transportation are explicable by differences in test-objects, circumstances and place of inoculation. The latter seems to influence the direction of the first virus-transportation.

Many authors have experimented with tobacco plants and the tobacco mosaic virus. Thung (1937) found that the higher the place of inoculation, the quicker the symptoms of virus will appear in the top. After inoculation of the lower leaves it is probably not the distance to the top that counts but possibly the age of the lower parts that causes a retardation of the reproduction of the virus and consequently a delay in transportation.

According to Zech (1952) a rapid virus-transportation takes place from an inoculated leaf at the stembase down to the roots. After a long time, usually 15 to 30 days, the young top-leaves become infected. The mature leaves at the stem are infected very late or not at all. When the place of inoculation is in the region of the stemtop, the virus also moves directly downwards.

Bennett (1940) inoculated one of the middle leaves of a tobacco plant with TMV and observed that virus-transportation took place both in apical and basipetal direction.

Samuel (1934) who inoculated a middle leaf of a tomato plant with TMV found virus-transportation down to the roots; shortly afterwards the virus moved from here to the top of the plant. When flowers or fruits were being formed, the virus was first transported in upward direction. Most authors agree that transportation occurs in a basipetal direction and that young shoots, flowers and fruits seem to attract virus.

Several authors have examined the influence of the removal of the stemtop or the axillary buds on virus-transportation. Grainger (1933) studied the influence exercised by the tops of the stems of tobacco plants. Leaves at the base of the stems were inoculated with TMV and, simultaneously, the top of the stem containing the vegetation-point, the leaf-primordia and the three youngest leaves, was cut from each plant. His results showed that the removal of the stemtop delayed the appearance of the virus-symptoms in the young leaves for two days and he concluded that the stemtop exercised an attractive action on the virus in the plant.

Bennett (1940) found that 20 to 50 days were required for the virus to cause symptoms in the top of the plant after inoculation of the rootsystems. In a second series of experiments plants, approximately 15 cm high, were cut back after inoculation of the roots leaving a stem about five cm long from which, on most plants, lateral buds soon developed into shoots. This removal had a decided accelerating

influence on the appearance of symptoms in the new growth of the lateral buds. The symptoms appeared 6 to 8 days after the tops were removed. So, contrary to Grainger's opinion, Bennett concluded that the removal of the stemtop exercised an attractive action on the virus present in the roots. It is probable that the newly developed young shoots were attracting the virus. VALLEAU (1941) observed the same phenomenon in Burley tobacco, inoculated with TMV at topping time.

ZECH (1952) experimented with tobacco plants, 100 to 130 cm high. The plants were maintained in a vegetative state by breaking off the stemtop and the topmost laterals before inoculation with TMV. Twenty-six days after the inoculation of the middle or upper leaf a newly formed lateral had replaced the original stemtop. At that time the plants were tested for the presence of the virus. It was found, that the newly formed lateral was first to show virus-symptoms. Later on the virus moved into some leaves at the stem. When all the axillary buds were removed, from these plants all of the leaves became infected. However, this last experiment is not described in detail and the influence of the topping on the very first spreading of virus from the inoculated leaf was not determined. From his experiments Zech concludes that as long as the new stemtop is not invaded by the virus, it seems to have a remarkable, directing action on the spread of the infection. When the stemtop is infected, the direction is altered but from Zech's description it is not clear how this occurs. The stemtop can become infected after inoculation of the rootsystem only when the roots keep their vegetation-points. When the latter are cut, no upward virus-transportation takes place until new vegetation-points have developed. This experiment is not described in detail either. Zech also stated, that the developing of flower-buds or maturing fruits seems to surpass the influence of the roottops.

Objections may be raised against this kind of experiments. Removal of the roottops altered more than one factor. The growth of the plants was temporarily disturbed by removal out of the pots. Wounds are made by removing the tops of the roots, stemtops or axillary buds. The metabolism of the plant and therewith the multiplication and transportation of virus were influenced by the removal of vital parts as root- and stemtops and axillary buds. Also GRAINGER (1933) based his suggestion that the stemtip influences virus-transportation in plants on the results of his experiments in which stemtops were removed. Removal of stemtops, however, includes removal of the leaf-primordia and at least one young leaf because the vegetation-point is completely surrounded by these parts. It is technically impossible to remove the vegetation-point and leave the other parts untouched (Fig. 1, Plate 1 C). By removal of the axillary buds, embryonic tissue and primordia are cut out. Excision of these parts alters more than one factor and the plant as a whole is physiologically changed.

2.2. MERISTEMS

Meristematic tissue occurs in different places in a plant, and has been divided into two groups by FITTING (1947) according to the place

of occurrence and the nature of its origin. The *primary* meristems come into being during the division of the germ cell and build up the multicellular embryo. During post-embryonic growth and development primary meristematic tissue occurs mainly in the stem, roottips and in axillary buds. The *secondary* meristems originate from mature cells that are undergoing change of function and being transformed into embryonic cells by cell-division. Wound-meristems and cork-cambium may be considered as secondary meristems. According to KONINGSBERGER (1943) it is difficult to draw a line between primary and secondary meristems.

Several authors have investigated the structure of the stemtip. BUVAT (1955) distinguished the following three zones in the apical- or top-meristem. 1) The apex in which cell-division seldom occurs during the vegetative state; 2) a central zone, the medullar meristem, with differentiated cells, and 3) a lateral zone, the initial ring, with a strong meristematic character, in which most cell-divisions occur and from which leaf-primordia arise. The fine structure of meristematic cells and their components has been studied and described by WHALEY, MOLLENHAUER & LEECH (1960).

The origin, the structure and the functions of the apical meristem of a tobacco plant were studied extensively by FARDY, CUZIN & SCHWARTZ (1953). As long as the top-meristem is actively dividing, differentiation of the pro-cambial tissue lying underneath occurs. When the terminal meristematic region ceases dividing an axillary meristem becomes active.

2.3. DISTRIBUTION OF VIRUS IN TOBACCO PLANTS

Remarkably accurate observations concerning the division of virus in an infected plant were made by BEYERINCK as early as 1898. According to this author, only those organs of the plants with tissues that are in an active state of growth and in which cell-division takes place, are attacked by the virus. Mature tissue did not seem to be affected, but it allowed the transportation of the virus.

Quantitative determinations of the virus-distribution in the top of the stem have been made by LIMASSET & CORNUET (1949). They found a low virus-concentration in the outmost smallest top-leaves and a high concentration in the young, vigorously growing leaves underneath. A maximum concentration was frequently found in one of the leaves at the fifth to the tenth place, reckoned from the vegetation-point. No virus was found in the top-meristem of a tobacco plant inoculated with TMV.

Unpublished experiments by A. F. Schippers-Lammertse have shown that the top-meristem and frequently the first two leaf-primordia of a tobacco plant inoculated with TMV remain virus-free while the young leaves underneath contain virus in a high concentration and leaves in advanced stages in development contain less virus.

ZECH (1952) obtained the same results from his experiments: in the young leaves virus was present in a higher concentration than in

leaves farther from the top of the stem. In his experiments also the top-meristem itself was virus-free.

3. THE INFLUENCE OF REMOVAL OF MERISTEMATIC TISSUE ON THE RATE OF VIRUS-TRANSPORTATION

3.1. Material and methods

Plants of the *Nicotiana tabacum* L. cultivar. Samsun were used in all experiments. This variety was chosen because inoculation with TMV is always successful and results in systemic infections. It was also important to these experiments that leaves, cut from these plants retained their turgor for at least three days when their bases were placed in water. Moreover, this variety was suitable for the cultivation of callus-tissue. All plants were cultivated in the greenhouse and were used when they were six weeks old, in a six-leaf stage, with a height of 4 to 6 cm. If otherwise it will be mentioned in the individual experiments.

All experiments were carried out with a strain of *Nicotiana* virus 1 Smith, that shows distinct symptoms on Samsun tobacco plants. Sap, pressed out of leaves of tobacco plants that were infected with the strain of TMV and showed distinct virus-symptoms, was used as inoculation-material.

A leaf of a Samsun plant was dusted with carborundum-powder and then inoculated with sap containing TMV. After about one minute this leaf was rinsed carefully with water, taking care that the leaf did not touch other leaves and that the rinsing water did not reach the soil or other parts of the plant. The lowest, the middle or the topmost leaf was chosen as the place of inoculation.

The presence of TMV can be demonstrated easily by means of the test-plant *Nicotiana glutinosa* L., which shows local lesions when inoculated with TMV.

The following method was used to establish the arrival of virus-material in a leaf of a Samsun tobacco plant. All the leaves of an inoculated plant were cut off at specifical intervals after inoculation. After each cut the knife was disinfected. Each leaf was placed with its base in a test-tube containing some water. Under this condition, virus-material already present in the leaves was allowed to multiply for three days (Plate 1 A). After this period each leaf was pressed out and the sap was tested for the presence of virus. For this purpose a leaf of *Nicotiana glutinosa* was dusted with carborundum-powder and inoculated with the sap. After rinsing with water the leaves were placed in a petri-dish with moistened filter-paper under fluorescent light with an intensity of about 1000 lux. After 48 hours the number of local lesions on the leaves was counted. When more than two local lesions were present on one leaf it was assumed that the sap contained virus. Generally the number of local lesions per leaf varied from 8 to more than 100. The sap of the rootsystems was tested for the presence of virus too.

From a number of plants the stemtips or the axillary buds were removed simultaneously with the virus-inoculation. The stemtip, with a length of 2 mm, contained the top-meristem, the leaf-primordia and 3 to 4 very young leaves (Fig. 1, Plate 1 B). The axillary buds contained the top-meristem, the leaf-primordia and sometimes one to two very young leaves.

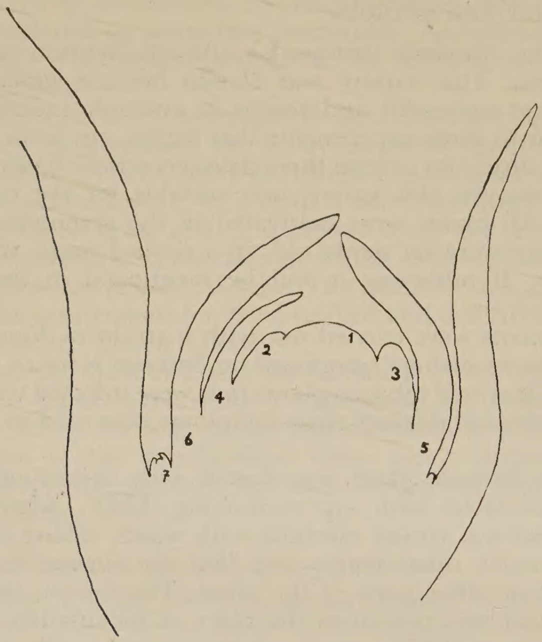

Fig. 1. Scheme of the stemtop of *Nicotiana tabacum* L. 1: youngest leaf-primordium. 2 and 3: older primordia with the beginning of the development of pro-vascular strands. 4, 5 and 6: young leaves in different stages of development. 7: axillary bud with leaf-primordia.

Wounding of a plant without removing tips or buds was achieved by gently scraping the epidermis of the stem either along the whole length at two opposite sides or in a circular three mm band circling the stem just under the stemtop. In this way only the cells of the epidermis were damaged or atmost the layer of parenchymatic cells just underneath, but not the more deeply located layers of cells.

3.2. Experiments on the influence of the place of inoculation on virus-transportation in the plant

To determine the influence of the place of inoculation on the rate of transportation of the virus, 18 plants in a four to six-leaf stage, six weeks old, were divided into six groups of three plants. Two groups were inoculated on the lowest leaf with sap containing TMV, two groups were inoculated on a middle leaf and two groups on the top-

most usable leaf. After 48 and 72 hours, respectively, the presence of virus in the leaves was determined by the local lesion method (Table I, exp. 1).

Table I

Influence of the place of inoculation on the rate of virus-transportation

Place of the inoculated leaf	Time after inoculation in hours	Virus-containing leaves		Ratio [3])		Virus-containing root-systems	
		exp. 1	exp. 2	exp. 1	exp. 2	exp. 1	exp. 2
lowest leaf [1])	48	1/11 [2])	2/13 [2])	0.08 ± 0.14	0.15 ± 0.11	0/3	0/3
,, ,,	72	4/12	6/12	0.33 ± 0.14	0.50 ± 0.00	0/3	1/3
middle leaf .	48	3/12	4/12	0.25 ± 0.00	0.33 ± 0.14	0/3	0/3
,, ,, .	72	6/13	8/12	0.47 ± 0.06	0.67 ± 0.14	0/3	0/3
topmost leaf.	48	1/14	1/11	0.07 ± 0.12	0.08 ± 0.14	0/3	0/3
,, ,, .	72	3/12	5/12	0.25 ± 0.00	0.42 ± 0.14	1/3	0/3

[1]) Three plants per group.

[2]) numerator: number of virus-containing leaves per group of plants excluding the inoculated ones; denominator: total number of leaves tested per group of plants excluding the inoculated ones.

[3]) Ratio of virus-containing leaves to ten tested leaves and standard-deviation. Second figure after decimal is approximate.

The number of virus-containing leaves of each group of tobacco plants is expressed in relation to the total number of leaves of the group tested. The inoculated leaves were not included, because they always contained virus. This ratio gives an indication of the spread of the virus through the plant. In a few cases only one leaf, and that at a large distance from the place of inoculation, contained virus. In such plants the ratio of virus-containing leaves is low, although the virus was transported a considerable distance. On the whole, however, the ratio of virus-containing leaves at a definite time appeared to be a good indication of the rate of virus-transportation.

Forty-eight hours after inoculation, of the blank number of leaves picked from plants inoculated on either the topmost or lowest leaf only one leaf contained virus. Of the blank number of leaves cut from the plants inoculated on a middle leaf, three leaves contained virus. Seventy-two hours after inoculation the number of virus-containing leaves was higher in all groups, the highest number was always reached in plants of which the middle leaf had been inoculated.

The fact that the number of virus-containing leaves per plant within one group can vary, makes it difficult to evaluate the differences stated between the groups. The standard-deviation of the ratio of virus-containing leaves of the plants within one group was calculated. In the experiments described in this paper, the differences between the groups were tested by Students "t"-test, as the number of testplants was rather small: three or five per group.

This experiment was repeated with six weeks old plants (Table I, exp. 2). In all groups the number of virus-containing leaves was somewhat higher than in the previous experiment. In this trial too, the inoculation of the middle leaf caused the highest rate of virus-transportation. From the two experiments it can be concluded that inoculation of a middle leaf of a plant causes the most rapid transportation of virus-material through the plant.

The fact that in practically all cases the rootsystems do not contain the virus, indicates that at least in our experiments the virus is not first transported to the roots before entering the leaves, as SAMUEL (1934) concluded.

3.3. COMPARISON OF THE RATE OF VIRUS-TRANSPORTATION IN PLANTS WITH AND WITHOUT A STEMTIP

The next experiment was intended to determine the influence of removing the stemtip simultaneously with the inoculation of a leaf. Though inoculation of the middle leaf seemed to be most promising, in this experiment the lowest and the topmost leaf were also used as place of inoculation in order to compare the results of this experiment with those of the former.

a) *Inoculation of the lowest leaf*

Thirty tobacco plants were inoculated on the lowest leaf with sap containing TMV. Of half this number the stemtip of two mm was removed simultaneously with the inoculation. The removal took place with the aid of a very sharp knife, in order to make the injury as slight as possible. The 15 plants without the stemtip were divided into three groups of five plants of which the number of virus-containing leaves was determined 48, 96 and 120 hours, respectively, after inoculation. The plants with stemtips were treated in the same way. In Table II A a survey is given of the place of the virus-containing leaves.

It was not uncommon for a virus-free leaf or leaves to be found between two virus-containing leaves. There was no correlation between the position of the leaf and the time of appearance of the virus.

Forty-eight hours after inoculation, the ratios of virus-containing leaves of plants with and without stemtip were 0.14 and 0.13, respectively (Table II B). The virus had not yet penetrated into the rootsystems. Ninety-six hours after inoculation a ratio of 0.74 and 0.63, respectively, was found. Two of the five rootsystems of the plants with a stemtip were infected and all of those of the plants without stemtips. One hundred and twenty hours after inoculation a high number of the leaves contained virus in both groups while three of the five rootsystems of the plants with a stemtip were still virus-free.

From this experiment it appeared that the ratios of virus-containing leaves of plants with and without stemtip were not distinctly different. Removal of the stemtip simultaneously with the inoculation of the lowest leaf, has little or no influence on the rate of virus-transportation to the higher leaves in the plant. The only difference between the

TABLE II

The spreading of virus after removal of the stemtip of plants compared with that in plants with intact stemtips 48, 96 and 120 hours after inoculation of the lowest leaf.

A. Place of the virus-containing leaves at the stem of plants with intact stemtip.

hours after inoculation	48					96					120				
Plant number [1]	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
6th leaf					–′	x′	–′						x′	x′	x′
5th ,,	–′	–′	–′	–′	–	x	x		x′	x′	x′	x′	x	x	x
4th ,,	–	x	–	x	x	x	x	x′	x	–	x	x	x	–	x
3rd ,,	–	–	–	–	–	–	x	x	–	x	–	x	x	–	–
2nd ,,	–	–	–	–	–	–	–	x	x	x	x	x	–	x	x
1st ,,	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
root-system	–	–	–	–	–	–	–	x	–	x	x	x	–	–	–

Place of the virus-containing leaves at the stem of plants without intact stemtip.

	48					96					120				
7th leaf		★		★	★										★
6th ,,	★	–		–	–	★		★	★	★	★				x
5th ,,	–	–	★	x	–	–	★	–	x	x	x	★		★	–
4th ,,	–	–	–	–	x	x	–	x	–	–	x	x	★	x	x
3rd ,,	–	–	–		–	–	x	x	x	x	–	x	x	x	x
2nd ,,	x	–	–	–	–	x	x	x	–	x	x	x	x	x	x
1st ,,	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
root-system	–	–	–	–	–	x	x	x	x	x	x	x	x	x	–

B. Calculation of the number of virus-containing leaves.

Presence of stemtip	Time after inoculation in hours	Virus-containing leaves	Ratio [3]	Virus-containing rootsystems
+	48	3/21 [2]	0.14 ± 0.13	0/5
+	96	15/21	0.74 ± 0.16	2/5
+	120	18/23	0.79 ± 0.14	2/5
—	48	3/22	0.13 ± 0.12	0/5
—	96	12/19	0.63 ± 0.13	5/5
—	120	15/17	0.91 ± 0.12	4/5

1) Five plants per group.
2) and 3) See note Table I.
′ stemtip.
★ stemtip removed.
• inoculated leaf.
– leaf or rootsystem without virus.
x leaf or rootsystem containing virus.

plants with and without a stemtip seemed to be a somewhat faster virus-transportation to the rootsystems after removal of the stemtip. It is possible that the lowest leaf as a virus-source is too far away from the stemtip, to be influenced by its presence or absence.

b) *Inoculation of the middle or the topmost leaf*

Forty plants were divided into eight groups of five plants. Of the plants of four groups the stemtips were removed simultaneously with the inoculation. Two groups of plants with and two groups without the stemtip were inoculated on the middle leaf and the other four groups on the topmost leaf that still could be inoculated. After 48 and 72 hours it was determined in which leaves virus was present (Table III).

TABLE III

Rate of virus-transportation after inoculation of the middle leaf or the topmost leaf of plants with and without stemtip.

Place of the inoculated leaf	Presence of stemtip	Time after inoculation in hours	Virus-containing leaves	Ratio [3])	Virus-containing rootsystems
middle leaf [1]) . . .	+	48	6/19 [2])	0.31 ± 0.06	0/5
,, ,, ...	+	72	12/19	0.63 ± 0.13	3/5
,, ,, ...	—	48	5/18	0.28 ± 0.04	0/5
,, ,, ...	—	72	13/19	0.68 ± 0.11	2/5
topmost leaf	+	48	2/24	0.08 ± 0.11	0/5
,, ,, ...	+	72	10/24	0.42 ± 0.04	2/5
,, ,, ...	—	48	1/18	0.05 ± 0.11	0/5
,, ,, ...	—	72	8/19	0.42 ± 0.12	3/5

[1]) Five plants per group.
[2]) and [3]) See note Table I.

The number of virus-containing leaves in the plants still in possession of the stemtip does not differ distinctly from that of the plants without stemtip: 48 hours after inoculation of the middle leaf the ratios were 0.31 and 0.28, respectively; 72 hours after inoculation the ratios were found to be 0.63 and 0.68, respectively. After inoculation of the topmost leaf the rate of virus-transportation was much lower than after inoculation of the middle leaf: a ratio of only 0.08 of the leaves of the plants with a tip and a ratio of 0.05 of those without the tip was found when tested 48 hours after inoculation. When tested 72 hours after that treatment the ratio was 0.42 for both groups of plants. The virus had not penetrated any of the rootsystems during the first 48 hours after inoculation. The number of infected rootsystems at 72 hours after inoculation did not appear to be influenced by the removal of the stemtip or the place of inoculation.

From the two experiments the conclusion could be drawn that inoculation of the middle leaf caused the most rapid virus-transportation to the other leaves of the plants. Therefore in the next experiments the middle leaf was always chosen as the place of inoculation.

In the preceding experiments there was no distinct difference in the rate of spreading of virus-material through the plant between plants with and those without the stemtip, neither in the transport to the leaves nor to the roots. The removal of the stemtip of a plant does not seem to influence the rate of virus-transportation out of the inoculated leaf whatever its place. No attracting or directing action of the stemtip could be demonstrated by removing this part of the plant.

3.4. Experiments on the influence of the removal of the axillary buds on the rate of virus-transportation

Although removal of the stemtip simultaneously with the inoculation does not influence the rate of spreading of the virus through the plant, it is possible that removing all the axillary buds does have an influence. To answer this question, the middle leaves of 24 plants were inoculated. The plants were divided into 4 groups of 6 plants. From the plants of the first group no axillary buds or stemtips were removed; from those of the second group the stemtips were cut out;

TABLE IV

Influence of removal of the stemtip and/or the axillary buds of plants on the rate of virus-transportation.

Experiment number	Group number [1]	Removal of	Time after inoculation in hours	Virus-containing leaves	Ratio [3]	Virus-containing root-systems
1	1	—	72	2/13 [2]	0.15 ± 0.13	0/3
			96	4/14	0.28 ± 0.10	0/3
	2	stemtips	72	2/11	0.17 ± 0.14	0/3
			96	4/12	0.33 ± 0.14	0/3
	3	axillary buds	72	5/12	0.42 ± 0.14	0/3
			96	8/12	0.67 ± 0.14	0/3
	4	tips and buds	72	5/10	0.50 ± 0.17	0/3
			96	8/12	0.67 ± 0.14	0/3
2	5	—	48	1/11	0.08 ± 0.14	0/3
			72	4/11	0.36 ± 0.12	0/3
	6	axillary buds	48	4/12	0.33 ± 0.14	0/3
			72	6/12	0.50 ± 0.00	0/3
3	7	—	72	2/12	0.17 ± 0.14	0/3
			96	4/14	0.28 ± 0.10	0/3
	8	axillary buds	72	6/13	0.47 ± 0.06	0/3
			96	10/13	0.77 ± 0.03	1/3

[1]) Three plants per group.
[2]) and [3]) See note Table I.

from those of the third group all the axillary buds; and from the plants of the fourth group both stemtips and axillary buds were taken away. All of these treatments were performed simultaneously with the inoculation.

From each group the presence of virus in the leaves and the rootsystems was tested 72 and 96 hours after inoculation (Table IV, exp. 1).

The virus had not penetrated the rootsystems. Seventy-two hours after inoculation the spreading of the virus in the plants of the first group with a ratio of virus-containing leaves of 0.15 did not differ distinctly from that of the second group with a ratio of 0.17. In the third and fourth groups the rate of virus-transportation was higher showing a ratio of 0.42 and 0.50, respectively. Ninety-six hours after inoculation the same picture occurred: removal of the stemtip did not influence the virus-transportation, removal of the axillary buds did. In all groups the virus went upwards and downwards. It could be concluded that removal of the axillary buds caused a higher rate of virus-transportation from the inoculated leaf to other leaves.

Two more experiments were carried out to investigate the influence of removal of all the axillary buds (Table IV, exp. 2 and 3). It was again shown that removal of the axillary buds caused a higher rate of virus-transportation.

It can be asked, however, which factors are of influence on the rate of virus-transportation. It is possible that the loss of meristematic tissues by removal of all the axillary buds exercises an effect, but it also may be that the effect is due to the wounds necessarily made, followed by wound-reactions. Perhaps a combination of these factors is responsible for the increased rate of virus-transportation.

4. INFLUENCE OF WOUNDING ON THE RATE OF VIRUS-TRANSPORTATION IN TOBACCO PLANTS

4.1. Literature

Each injury, inflicted upon a living plant, causes changes in the damaged cells or tissues. These changes can be confined to the immediate neighbourhood of the wound or they may be perceptible at a great distance from it. The influence may last only a short time after the injury or for many weeks or months (Kuster, 1925).

When herbaceous plants are injured the cells at the border of the wound die off. Beneath the dead cells a secondary meristem or wound-meristem may be built, though only mature cells have been wounded (Went, 1923).

Secondary meristems originate from mature living cells, which become meristematic under the influence of a wound. They form a layer of initiating cells amid uninjured parenchymatous tissues adjacent to the wound. The cell-divisions take place parallel to the wound-surface. The newly formed tissue, the wound-periderm, commonly consists of three layers of tissue: the initiating layer or meristem, known as the phellogen or cork-cambium; the layer of cells

formed by this meristem towards the outside, the phellem or cork; and, usually, a layer formed toward the inside, the phelloderm. (Eames & Daniels, 1925). The cell-walls of the periderm have a coat, the cork- or suberin-lamellae, consisting of a lipophillic substance (Frey-Wyssling, 1959). One of the special functions of the periderm is the protection of wounds through the production of wound-cork. Tissue dead from any cause is usually shut off from that which is healthy by such a periderm layer (Eames & Daniels, 1925).

Often other processes take place in the living cells adjacent to the wound. The cell-walls of the cells bordering the wound are lignified and cork lamellae are deposited against the walls. These processes, called meta-cutinisation, are very common in plants after wounding. In this way an enclosure of the wound-surface of a potato tuber may be formed as early as 12 hours after injuring.

The initial cells of the secondary meristem may also produce callus-tissue. External conditions are of great influence on the method of wound-healing; if humidity is low, periderm is formed. In that case cell-multiplication progresses slowly, the new cells differentiate soon and the cells just beneath the wound-surface become corky. If humidity is high, hypertrophy and cell-division occurs, the multiplication of the cells proceeds at a high rate and the great mass of parenchymatous or meristematic cells are considered as callus. Only the walls of the cells at the surface of the new tissue are suberized, especially when the outer layers are dying off (Fitting, 1947). There is, however, no clear cut difference between wound-periderm and callus-tissue.

The reversion of mature cells to the juvenile state is ascribed to the action of wound-hormones, produced by the wounded cells (Haberlandt, 1921; Scheer, 1953). Also the intact cells surrounding the wound produce substances of influence on the formation of hypertrophic cells and on the occurrence of cell-divisions. They are produced only during a definite period after injuring. When the wound-surface is rinsed with water for ten hours, no callus-tissue is built afterwards. The cells under the wound-surface are, however, capable of reacting when after the rinsing period, wound-sap is put on the wound-surface. In that case callus-tissue is formed (Braun, 1957).

Wound-hormones present in the wound-sap of plants not only effect the morphology of resting cells, but provoke: 1) an increased respiration (the so called wound-respiration), 2) an increased metabolic activity, 3) changes in the permeability of the protoplast, and 4) an accelerated rate of protoplasmic-streaming. The latter was observed by Zech (1952), who studied the influence of injuring a hair on a tobacco leaf. By cutting the tip-cell of the hair the rate of the protoplasmic-streaming in the other cells was increased immediately after the injury. Fifteen hours later the rate of flow was normal again.

It was shown by Braun (1952) that the condition of the cells beneath the wound-surface is determining for the occurrence of a tumor in the presence of virulent crowngall bacteria, *Agrobacterium tumefaciens* (Sm. et Towns.) Conn. The tumor is formed only when the virulent bacteria come into contact with cells activated by wound-substances.

This "state of conditioning" occurs 24 to 96 hours after wounding. Conditioning takes place gradually, it reaches a maximum during the period from 48 to 72 hours after a wound has been made. The activation is proportional to the concentration of wound-substances. The first 48 hours after wounding seem to be important for the activation of the cells. It is at this stage that the cells show a high rate of metabolic activity. The stage of "conditioning" passes into a state in which microscopically visible wound-reactions occur. After this period the healing process has advanced too far and the bacteria are no longer able to induce tumor-formation.

4.2. Wound-reactions in Nicotiana tabacum L. cultivar. Samsun

It may be asked which changes are effected in the tissues of a tobacco plant, when the axillary buds or the stemtips are removed or when the surface of the stem is slightly wounded.

To answer this question, transverse sections of the wound-region were made at different times after wounding of the stem or removal of the stemtip and the axillary buds. The sections were stained with ammoniacal gentian-violet-solution and differentiated in a hydrochloric acid-solution in water. In this way only suberin- and cork-containing cell-walls were stained. According to Schömmer (1949) this staining is more sensitive than the staining with Sudan III.

Twenty-four hours after wounding of the stem-surface the beginning of the coating of suberin against the inner-side of the walls of the intact cells beneath the wound-surface was perceptible. After 48 hours the suberin-layer was more intensively stained. During the next 72 hours

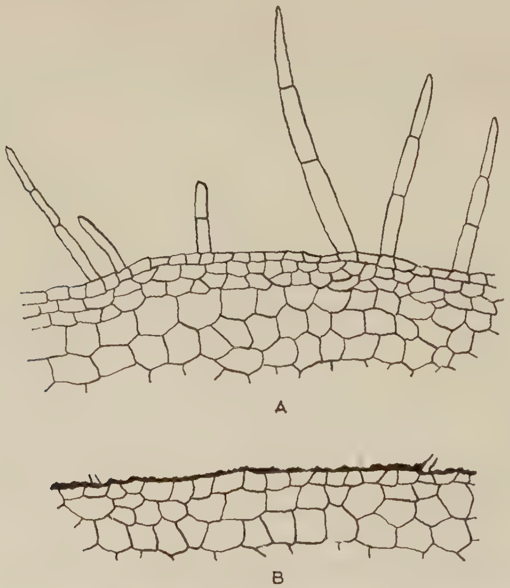

Fig. 2. Transverse section of stems of *Nicotiana tabacum* L. stained with ammoniacal gentian-violet-solution. A: unwounded; B: 48 hours after wounding.

this condition remained unchanged and after this period the observations were stopped.

These results indicate that here a simple process of wound-healing had taken place. The damaged cells died, became brown and parched, and within 48 hours the walls of the underlying cells were coated with a suberin layer. The same process occurred after removal of the stemtip or the axillary buds. The building of a secondary meristem or formation of callus-tissue has never been detected. No microscopically visible changes were observed later than 48 hours after wounding (Fig. 2). There was no difference in the way of wound-healing between the healthy plants and the plants infected with TMV, under the same conditions.

It was considered possible, that during the first 48 hours after wounding, in which period the wound-reactions occur, the active state of the cells is of influence on the direction and the rate of the virus-transportation in the plant. To investigate this possibility, additional experiments were carried out.

4.3. Influence of wounding of the stem on the rate of virus-transportation

In the following experiments, the influence of removing the axillary buds and the stemtip on the rate of virus-transportation was compared with that of wounding of the surface of the stem.

The experiment was carried out with four groups of three plants. In the first group no wounding or removal took place; in the second group all the axillary buds and the stemtips were removed; in the third group a slight circular wound was inflicted, surrounding the stem just under the stemtop; and in the fourth group the stem was slightly damaged by injuring the epidermis along its whole length at two opposite sides. Simultaneously, a middle leaf of all plants was inoculated. The plants were tested for the presence of the virus, 120 hours after the inoculation (Table V A and B).

The virus could not be detected in any of the rootsystems. In the first group the ratio of infected leaves was only 0.35. In the second group the ratio was 0.67, an increase which was in agreement with the results of the preceding experiment (p. 125). In the third group the ratio was found to be 0.43. Wounding of the stem just under the top without removal of the stemtip appears to have as little influence on the virus-transportation in the plant as a wound made by the removal of the stemtip. It is remarkable that here the virus was transported only in upward direction out of the inoculated leaf. In the fourth group the ratio of infected leaves was highest, namely 0.81. Thus it appears that the wounds of the epidermis inflicted along the whole length of the stem have a greater influence on the rate of virus-transportation than the very small circular wound under the stemtop, and somewhat more than removal of all the axillary buds.

In a following experiment this was further confirmed: Twenty-five plants were divided into five groups of five plants, all six weeks old. In the first group no buds were removed or wounds inflicted. In the

TABLE V

Spreading of virus in plants after removal of the stemtip and the axillary buds or wounding of the stem, 120 hours after inoculation of the middle leaf.

A. Place at the stem of the infected leaves.

Group number [1]	1			2			3			4		
Treatment	Controls			Stemtips and axillary buds removed			Circular wound under the stemtop			Wounding of the stem along its whole length		
plant number . . .	1	2	3	1	2	3	1	2	3	1	2	3
										x′		
6th leaf	—′		—′	★	★	★		x′	x′	x	x′	—′
5th ,, ...	—	—′	x	—	—	x	x′	x	—	x	x	x
4th ,, ...	x	—	x	x	x	x	x	—	x	x	x	x
3rd ,, ...	●	x	●	●	●	●	●	●	●	●	x	●
2nd ,, ...	x	●	—	x	—	x	—	—	—	x	●	x
1st ,, ...	—	—	—	x	x	—	—	—	—	—	—	x
rootsystem	—	—	—	—	—	—	—	—	—	—	—	—

B. Number of infected leaves in relation to the total number of leaves per group

Group number [1]	Virus-containing leaves	Ratio [3]	Virus-containing rootsystems
1	5/14 [2]	0.35 ± 0.09	0/3
2	8/12	0.67 ± 0.14	0/3
3	6/14	0.43 ± 0.06	0/3
4	13/16	0.81 ± 0.02	0/3

[1] Three plants per group.
[2] and [3] See note Table I.
Explanation of symbols, see Table II.

second group the stemtips were removed and in the third group all the axillary buds. In the fourth group a small circular wound was made just under the stemtop and in the fifth group the stems were wounded along their whole length at two opposite sides. These treatments took place simultaneously with the inoculation of the middle leaf. One hundred and twenty hours after this treatment the leaves and the rootsystems were cut off and tested for the presence of virus (Table VI A and B).

In this experiment the number of virus-containing leaves was smaller than in the preceding one. However, in all groups the virus had penetrated into the roots. In the controls the ratio of infected leaves was only 0.10. In all the other groups this ratio was higher; in the second group 0.28. Here the virus-transportation took place mainly in an upward direction. In the third group the ratio was 0.55. So here a great virus-spreading had taken place and both upward and downward. In the fourth group a ratio of 0.35 was found; this

TABLE VI

Spreading of virus in plants after removal of the stemtip, the axillary buds or wounding of the stem, 120 hours after inoculation of the middle leaf.

A. Place at the stem of the infected leaves.

Group number [1]	1					2					3					4					5				
Treatment	Controls					Stemtips removed					Axillary buds removed					Circular wound under the stemtop					Wounding of the stem along its whole length				
Plant number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
6th leaf. .		–′				★	★		★																
5th ,, . .		–		x′		x	x	★	x	★	–′	–′	x′	–′	x′	x′	–′	x′	–′	x′	–′	x′	x′	x′	x′
4th ,, . .	x′	–	–′	–	–′	–	–	x	–	x	x	x	–	x	–	x	x	–	x	x	x	x	x	x	–
3rd ,, . .	–	●	–	●	–	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
2nd ,, . .	●	–	●	–	●	–	–	–	–	–	–	x	x	x	x	–	–	–	–	–	x	–	–	–	x
1st ,, . .	–	–	–	–	–	–	–	–	–	–	x	x	–	–	–	–	–	–	–	–	–	x	–	x	x
rootsystem.	–	x	–	–	x	–	–	–	x	–	–	x	x	–	x	x	–	–	x	–	–	–	–	x	x

B. Number of infected leaves in relation to the total number of leaves per group.

Group number [1]	Virus-containing leaves	Ratio [3]	Virus-containing rootsystems
1	2/18 [2]	0.10 ± 0.14	2/5
2	5/18	0.28 ± 0.04	1/5
3	11/20	0.55 ± 0.11	3/5
4	7/20	0.35 ± 0.14	2/5
5	13/20	0.65 ± 0.14	2/5

[1] Five plants per group.
[2] and [3] See note Table I.
Explanation of symbols, see Table II.

ratio does not differ markedly from that of the second group. These results confirm those of the preceding experiments, which showed little difference between stemtip-removal and circular injury below the stemtop. In the fifth group the virus-spreading was greatest with a ratio of 0.65. These results indicate that both wounding of the stem along the whole length and wounding by removing all the axillary buds accelerate the speed of virus-transportation in a tobacco plant.

It is thus impossible to determine the influence of the stemtips or the axillary buds on the virus-transportation in a plant by removing these plant-parts, for by removing these meristematic tissues, wounds are necessarily made. Probably the wounding, followed by wound-reactions has such an influence on the virus-transportation that a possible effect of the stemtips or the axillary buds cannot be demonstrated.

4.4. Influence of different phases of wound-healing on the rate of virus-transportation

From the preceding experiment it became evident that a wound exercises an attractive action on virus-material present in the plant. Tissues in a phase of wound-healing may influence the speed with which virus moves through a plant.

A question is whether the wound in all the different phases of healing exercises an attractive action. From histological observations in tobacco plants it became evident, that only during the first 48 hours after wounding microscopically visible changes occurred in the cells under the wound-surface (p. 129). Braun (1952) from his experiments with *Agrobacterium tumefaciens* concluded that the wound-reactions occur immediately after injuring and that they last longer than 48 hours. Wounded cells of tomato plants were found to be in an activated state from 24 to 96 hours after wounding. The state of conditioning reached the highest level during the period of 48 to 72 hours after wounding. It is then possible, that abnormal physiological processes caused by wounding, though microscopically not visible may occur also in cells of the tobacco plant, activating them over a period of more than 48 hours (p. 128).

It was therefore important to determine the influence of wounding in tobacco plants on the rate of virus-transport for longer than 48 hours after damaging the epidermis.

In the following experiments the stems of the plants were wounded from 48 hours before to 72 hours after inoculation in order to determine the influence of the different phases of wound-healing on the speed of virus-transportation in a tobacco plant.

The experiments were carried out with seven groups of three plants. Plants of one group served as a control and were not wounded. Those of the other six groups were wounded by injuring the stem along its whole length at two opposite sides, 48 and 24 hours before, simultaneously with, 24, 48 and 72 hours after the inoculation of the middle leaf, respectively. The leaves were tested for the presence of virus 96 hours after the inoculation in the manner described (Table VII).

It became evident that the number of virus-containing leaves of the control plants did not differ markedly from the plants, wounded 48 hours before the inoculation; the ratio being 0.24 and 0.27, respectively. Probably the cells surrounding the injured area of the plants wounded 48 hours before inoculation were no longer in an activated state. Wound-healing may have been completed or nearly so. The ratio of 0.35 which was found in plants wounded 24 hours before inoculation, is of only slight statistical significance in differing from the control plants. When virus-transportation started in these plants, the cells in the neighbourhood of the wound were possibly no longer in the period of optimal activity. The activity was, however, still great enough to cause a very slight increase in the rate of virus-transport. Wounding simultaneously with, or 24 hours after, the inoculation resulted in an increased rate of virus-transportation, as

TABLE VII

Spreading of virus in plants, 96 hours after inoculation of the middle leaf and after wounding of the stem along the whole length at different times.

Experiment [1])	Moment of wounding with respect to the moment of inoculation	Virus-containing leaves	Ratio [3])	Virus-containing rootsystems
A	controls, not wounded . . .	4/16 [2])	0.24 ± 0.07	0/3
B	48 hours before inoculation .	5/18	0.27 ± 0.10	0/3
C	24 ,, ,, ,, .	6/17	0.35 ± 0.04	0/3
D	simultaneously with ,, .	8/18	0.44 ± 0.10	0/3
E	24 hours after ,, .	8/17	0.47 ± 0.06	1/3
F	48 ,, ,, ,, .	6/16	0.38 ± 0.04	0/3
G	72 ,, ,, ,, .	4/17	0.23 ± 0.09	0/3

[1]) Three plants per group.
[2]) and [3]) See note Table I.

the ratios were 0.44 and 0.47, respectively, 96 hours after inoculation. The tissues were probably in a period of optimal activity at the time the virus-material reached the attraction-sphere of the wounds. In plants wounded 48 hours after inoculation with a ratio of 0.38 the influence of the injuries was smaller, but significant different from that of the control plants. The plants wounded 72 hours after inoculation showed a ratio of 0.23, not differing from the controls. In this experiment possibly the virus-material had already started to move through the plant before the injuries were inflicted.

From these experiments it can be concluded that wounding seems to influence the speed of virus-transportation only when injuries are made within the period 24 hours before to 48 hours after inoculation of a middle leaf.

It may be asked, how much time the virus-material needs to enter the attraction-sphere of a wound of the stem after a leaf has been infected with the virus.

No virus can be demonstrated during the latent period, the first 30 hours following inoculation of a tobacco leaf with TMV. Virus-multiplication begins probably six to seven hours after the establishment of the infection (SIEGEL c.s., 1957; YARWOOD, 1952; KASSANIS, 1959). During the latent period, the virus-concentration is probably lower than 10^{-16} gram TMV/ml (SCHRAMM c.s., 1958).

In the literature, different data are found concerning the moment at which the newly built virus-particles start moving out of the inoculated leaf and reach other parts of the plant. Several authors have shown that the TMV or the infectious agents seldom begin to move out of an infected leaf earlier than 48 hours after inoculation (CAPOOR, 1949; KUNKEL, 1939; BEEMSTER, 1958). This was confirmed in our experiments (p. 123). It seems then reasonable to assume that virus-material enters the attraction-sphere of wounds approximately 48 hours after inoculation.

The wounded cells seem to influence the rate of virus-transportation

out of the inoculated leaf only between the moment of wounding and somewhat over 72 hours afterwards. This period coincides with the period of optimal activity of the conditioned cells mentioned by Braun (1952, Brants, 1961).

These experiments show that not only cells of tomato plants, but also those of tobacco plants are activated by wounding, though this fact has not been confirmed by microscopical observations. Also Braun could not observe microscopical or cytochemical differences between normal and conditioned cells.

It may be asked, what influence the removal of the axillary buds has on the rate of virus-transportation in a plant, when the removal is made at different times with respect to the moment of inoculation of a middle leaf.

In order to determine the effect both of removal of the axillary buds and of the wounding made by this removal, the following experiments were carried out. Twenty-one plants were divided in seven groups of three plants. One group served as a control and no axillary buds were cut out. In the other six groups all the axillary buds were removed at different times with respect to the moment of inoculation of a middle leaf, namely 48 and 24 hours before, simultaneously with, and 24, 48 or 72 hours after inoculation, respectively. The plants were tested for the presence of virus 96 hours after the inoculation (Table VIII).

Table VIII

Spreading of virus, 96 hours after inoculation of the middle leaf and after removal of the axillary buds of the plants at different times.

Experiment [1])	Moment of wounding with respect to the moment of inoculation	Virus-containing leaves	Ratio [3])	Virus-containing rootsystems
A	controls, not wounded . . .	4/14 [2])	0.28 ± 0.10	0/3
B	48 hours before inoculation .	4/13	0.31 ± 0.09	0/3
C	24 ,, ,, ,, .	6/12	0.50 ± 0.00	0/3
D	simultaneously with ,, .	10/14	0.72 ± 0.10	1/3
E	24 hours after ,, .	9/14	0.65 ± 0.09	1/3
F	48 ,, ,, ,, .	7/12	0.58 ± 0.14	0/3
G	72 ,, ,, ,, .	4/14	0.28 ± 0.10	0/3

[1]) Three plants per group.
[2]) and [3]) See note Table I.

The ratio of virus-containing leaves of the control plants (0.28) did not differ from that of the plants from which the axillary buds were removed 48 hours before inoculation (0.31). From the preceding experiment it appeared that wounding 48 hours before inoculation produced no perceptible effect (Table VII) and thus it may be assumed that the absence of the axillary buds had no effect either. Removal of the axillary buds within a period of 24 hours before to 48 hours after inoculation caused an increased rate of virus-transporta-

tion from the inoculated leaf to the other leaves, with a maximum when removal took place simultaneously with inoculation.

Results of these experiments agreed with the results of the previous experiments in which the stem was wounded. Again the highest increase of the rate of virus-transport occurred when the beginning of transportation (48 hours after inoculation) coincided with the period

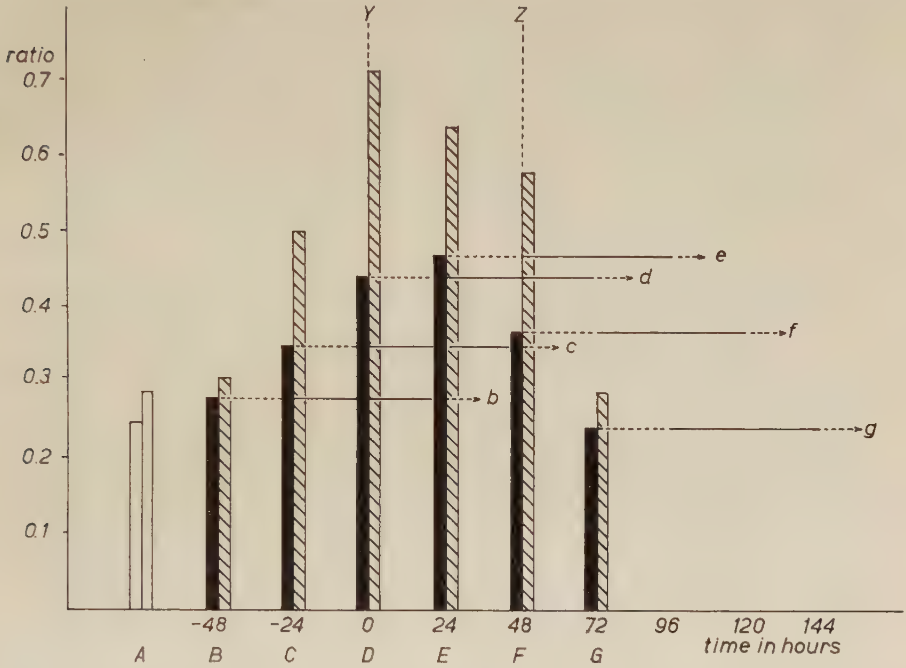

Fig. 3. Effect of wounding at different times with respect to the moment of inoculation on the rate of virus-transport. Abscis: time in hours with respect to the moment of inoculation. Ordinate: ratio of virus-containing leaves. Y: moment of inoculation. Z: begin of virus-transport out of the inoculated leaf.

□ : ratio of virus-containing leaves in plants without wounding or removal of axillary buds.

■ : ratio of virus-containing leaves after wounding of the stem.

▧ : ratio of virus-containing leaves after removal of the axillary buds.

——····→ : period of conditioning of the experiment concerned.

of optimal activity of the wounded cells. The trials showed that at least in this experiment removal of the axillary buds had a greater influence than wounding of the stem (Fig. 3). It is likely, that injuries inflicted over a great surface or upon many places have a greater influence on the rate of virus-transportation than small wounds and that it was not the absence of the buds but the wounding that caused these effects. So removal of all the axillary buds or wounding the stem along the whole length will have a greater influence than removing a stemtip or a circular wounding of the stem under the top.

From the last two groups of experiments it can be concluded that wounding with or without removal of the axillary buds influences the beginning of the virus-transportation out of the inoculated leaf only when the injury is inflicted within a period of 24 hours before to 48 hours after inoculation. It is unknown whether there is an increased food-transportation in the direction of the wounded cells during the conditioning phase. If this does occur, an increase in virus-transportation may be expected if virus-material moves together with nutrients. Healed wounds would not exert this influence.

It appears probable that the influence of a wounded area is limited to a local action, since removal of the stemtip did not influence the rate of virus-transportation. Therefore it was desirable to determine the influence of cells in a state of conditioning caused by localized wounding. The following experiments were performed with leaves in order to investigate the extent of this influence.

5. INFLUENCE OF WOUNDING ON THE RATE OF VIRUS-TRANSPORTATION IN A TOBACCO LEAF

5.1. Literature

Zech (1952) has investigated extensively the spread of virus in an inoculated leaf. The increased rate of streaming of the protoplasm, occurring in the cells after inoculation of a hair, was considered to be an indication that the virus or the infectious agent had reached these cells. In leaf-hairs wounded but not inoculated with virus, an increased rate of protoplasm-streaming was also observed, but became imperceptible after 20 hours. It can be asked, however, whether this accelerated speed of streaming is really an indication for the rate of spreading of the virus-material, as it is possible that this material passes through the cells without causing microscopically visible changes. Zech assumes that only after the passage of non-infective precursors of the virus-nucleoproteins, or other substances, streaming of the protoplasm in the cells is accelerated. He observed that six hours after inoculation the protoplasm-streaming in the upper- and under-epidermal cells was increased. In the upper-epidermis the infection first extended slowly to all sides. When the epidermal cells lying over the nerves were reached, the extension took place faster, parallel with the nerves. In a direction perpendicular to the veins, the rate of virus-transportation was about five times less. The infection in the parenchyma of a leaf extended first in all directions just as in the upper-epidermis. Virus-material follows a nerve in apical and basal direction, but more quickly in basipetal direction. During the first two days after inoculation, no infectious material was detectable in the leaf by means of the local lesion test on *Nicotiana glutinosa* L. The third day after inoculation the first inclusion bodies appeared and virus-material could be demonstrated in the expressed leaf-sap. From the fourth day on the number of local lesions increased.

To determine the effect of another kind of wounding, squeezing

the leaf cells, on the number of local lesions formed after inoculation with virus, YARWOOD (1953) pressed bean leaves at 20–240 lbs of pressure per square inch immediately before or immediately after inoculation with TMV. This treatment increased the number of local lesions, whether or not carborundum was used in the inoculation process. The increased infection due to pressing was greatest in leaves which had become somewhat resistant due to age. No effect on infection was observed if the leaves were pressed a day or more before or after inoculation. Inoculation by spraying with a virus-suspension without carborundum was successful immediately after pressing the leaf, but not without pressing. From these experiments it may be concluded that wounding of the leaf results in an increased virus-multiplication.

A disadvantage of the use of leaves to determine the rate of virus-transport is the fact that a leaf is not composed of a homogeneous tissue. HOLMES (1932) suggested that TMV does not generally utilize small veins in its movement in leaves of tobacco plants. When cuts through large veins just below the point of infection were made, the infection of the remainder of the plant was delayed for a short time. Leaves in which cuts were made in intervenal tissues but not through large veins showed less delay in transportation. Wounding by cutting big veins influences the rapid transport of virus-material through these nerves, but probably not the slow transportation from cell to cell in the parenchyma and in the epidermis (KÖHLER, 1950). It may be possible, however, that the conditioned cells in a leaf after wounding are capable of influencing virus-transportation in both the leaf-parenchyma and the nerves.

To examine the influence of cells in a stage of conditioning on virus-transportation in a leaf, the following experiments were carried out with tobacco leaves.

5.2. EXPERIMENTS WITH INJURED, INOCULATED LEAVES

The experiments with leaves were carried out to determine the influence of a slight wounding of a part of a tobacco leaf on the rate of virus-transportation inside this leaf after another part of the same leaf had been inoculated. These experiments can be performed with leaves still on the plant, or with cut leaves. The advantage of attached leaves is, that no extra injury at the petiole is made and the transportation in the leaf influenced by artificial wounding of the leaf-surface can be compared with the transport in unwounded leaves. The disadvantage of experiments with leaves in situ is that transportation in the leaf is influenced by factors lying in the plant itself, such as a tendency to basipetal transport or the influence of the axillary bud near the leaf. Operating with cut leaves, though more easy than with leaves in situ, also has disadvantages. An extra wounding of the petiole must be made and the metabolism of a cut leaf is not comparable with that of a leaf still attached to the plant.

First experiments were performed with cut leaves and leaves in situ to investigate whether the results from these experiments would be

comparable. In all of the following experiments the middle leaves of one plant or comparable leaves from different plants, six weeks old, were used. Defined strips two cm wide and perpendicular to the midrib of attached and cut leaves were inoculated with sap containing TMV. For this treatment a strip at the base, in the centre or at the top of the leaf was chosen. Some leaves were wounded by damaging the epidermal cells and hairs of a second two cm wide strip simultaneously with the inoculation. After inoculation the leaves were plunged into soap-water and then rinsed. The rinsing method appeared to be satisfactory, as virus could never be demonstrated (local lesion-test) in the water used for rinsing. Cut leaves underwent the same treatment as the attached leaves. The cut leaves were placed in a tube containing a half strength Knop-solution. Twenty-four hours after inoculation all leaves were cut perpendicular to the midrib in as many two cm strips as possible. The borders of the wounded and the inoculated areas coincided always with the borders of the strips. After each cutting the knife was disinfected. The leaf-strips were placed in a petri-dish on diluted Knop-solution where they stayed for the next 96 hours. During this period the leaves remained green and fresh and the virus-material present in the cells at the moment of cutting multiplied and became infectious.

Through the use of this technique, as opposed to that of Zech (1952), the rate of transportation of virus-material still not infectious in a leaf can be investigated without microscopic examination of the cells. After 96 hours each strip was pressed out, the sap was rubbed on leaves of *Nicotiana glutinosa*, and two days later local lesions were counted. When more than two lesions were present on one leaf it was assumed that the sap had contained virus. Twenty-four hours after inoculation of a strip at the top, the middle or the base of a leaf, the presence of virus-material in the other leaf-strips was ascertained by the method described above. In many cases, the spreading of virus-material out of the inoculated region had taken place. In Fig. 4, each leaf-diagram represents a combination of three leaves, thus, Figs. A1, A2 and A3 show the spread of virus in nine leaves in situ. Figs. B1, B2 and B3 show that of nine cut leaves, inoculated in the same way. In attached leaves the virus-spread 24 hours after inoculation at the top of the leaf seemed to be somewhat less than in cut leaves (A1 and B1). After inoculation of the middle part the same rate of virus-transport was found in both types of leaves (A2 and B2). Cut leaves inoculated at the bases gave no evidence of virus-transportation, while similarly inoculated attached leaves showed virus-spreading comparable to that observed in leaves inoculated at the top (A3 and B3). It was, however, impossible to detect clear cut differences in the rate of transport between cut leaves and leaves in situ. Therefore the experiment illustrated in Figs. C1, C2 and C3, was performed with cut leaves. Leaves were slightly wounded at different places simultaneously with inoculation of the centre strip. Comparison of diagrams B and C shows that wounding resulted in a greater virus-spread, when tested 24 hours after inoculation. The transport occurred

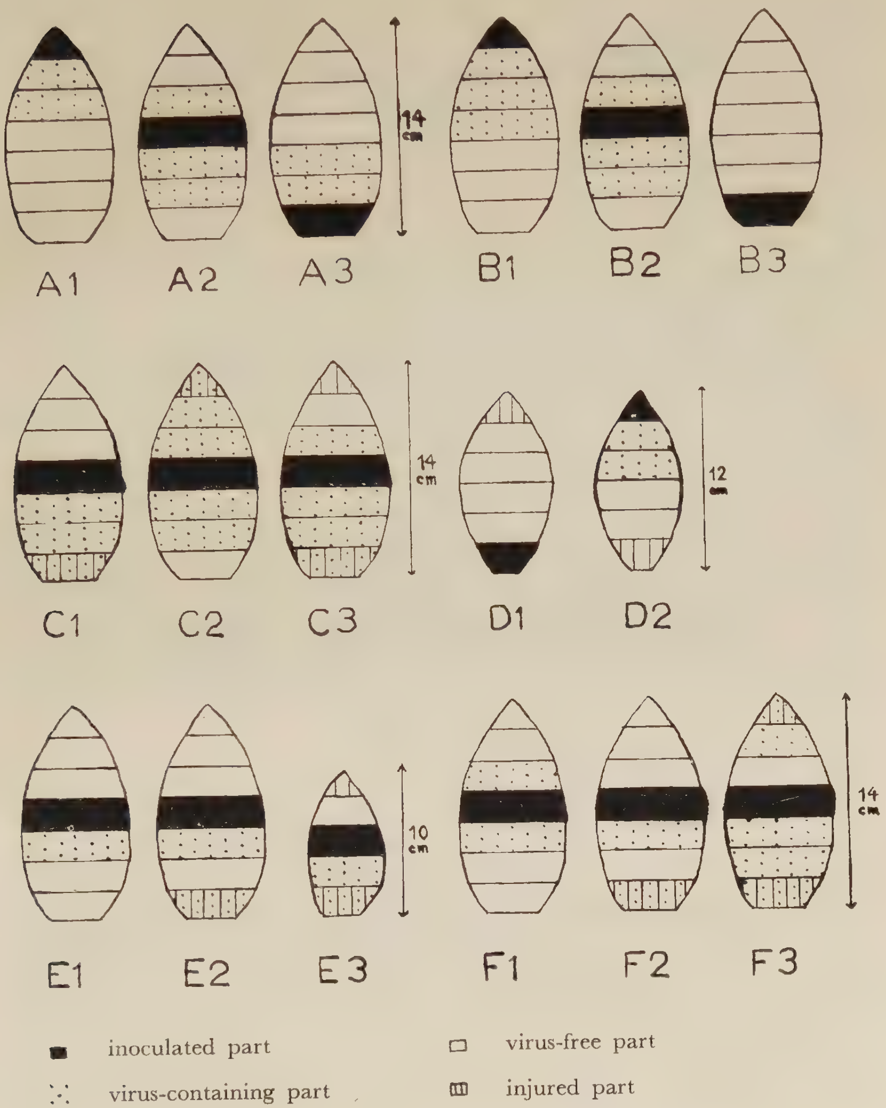

Fig. 4. The way of virus-spreading in leaves. Each diagram represents an average of 3 leaves. A: attached leaves. B to F: cut leaves. A, B, C and D: virus-spreading 24 hours after inoculation. E: spreading after 20 hours and F: spreading 22 hours after inoculation. Injuries were inflicted simultaneously with inoculation.

mainly in the direction of the wound. The effect of wounding at the base seems to surpass the influence of injuring the top (C3). Comparison of diagrams B and D shows that wounding of the top (D1) or of the base (D2) simultaneously with inoculation at the base or at the top, respectively, did not influence transportation of the virus-material. Perhaps the injured area was too far away from the inoculated region to exercise any influence. Since the virus-material was not attracted over great distances and since inoculation of the middle strip resulted in the highest rate of virus-transportation, the middle strip was always chosen as the place of inoculation in the experiments illustrated by diagrams E and F. In both of these experiments one set of three leaves was not wounded, one set was wounded at the base and one was wounded at both top and base. Results were recorded at 20 hours after inoculation in the experiment illustrated in diagram E and that of 22 hours in that represented by diagram F. In all cases wounding caused a more rapid transport of virus-material. In the leaves of diagram E2, F2 and F3 a virus-free region existed between two virus-containing strips. A virus-free region is shown in a diagram only when it was found in at least two of the three leaves in any set. It is possible that the virus-material is transported so quickly along the midrib that no virus-material is left in the virus-free zone between two virus-containing strips. In the former experiments illustrated by diagrams C and D, no interjacent virus-free zones were found 24 hours after inoculation. It may be that such zones are infected by diffusion later as the result of slow penetration of the virus-material in the leaf-parenchyma (Köhler, 1950). In diagram F3, after wounding of both top and base, the virus-material had already reached a great deal of the leaf in the 22 hours after inoculation.

From these experiments it can be assumed that there is some attractive action of a wounded area on virus-material transported in a leaf. It is, however, unknown whether a correlation exists between the period during which virus-material is attracted towards a wound and the period during which the wounded cells are in a state of conditioning. In order to answer this question and, at the same time, to investigate a possible influence of cutting the leaves before treatment, the following experiments were carried out with both attached and cut leaves. It had been found that strips smaller than two cm could be kept fresh during the 96 hours necessary for virus-multiplication and that the width of the strips could be reduced from 2 to 1 ½ cm. In the experiment with cut leaves, the leaves were removed from the plants immediately after inoculation of the middle strip. Injuries were inflicted upon the base of the leaves at 72, 48 and 24 hours before inoculation, respectively, and simultaneously with the inoculation.

These experiments were only partly comparable with the preceding experiments with whole plants, in which stems were wounded from 48 hours before to 72 hours after inoculation. This change in method was essential since it was known that the virus-material could penetrate the whole leaf in 24 hours and in order to detect attraction by the wounded areas, the leaves had to be divided into strips at 20 and 22

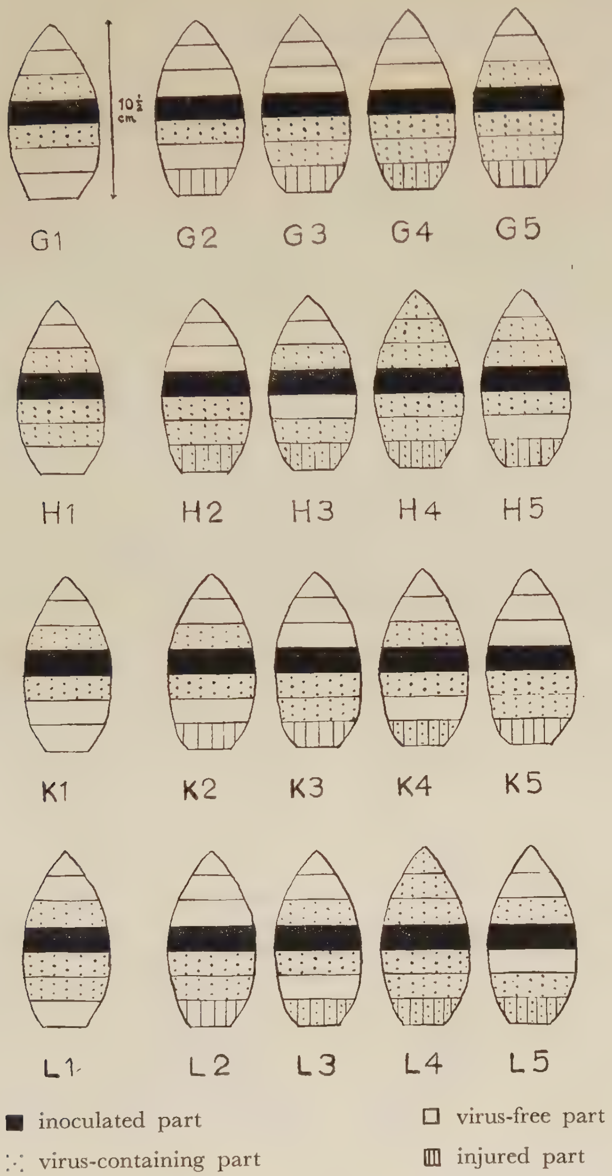

Fig. 5. The way of virus-spreading in leaves. Each diagram represents an average of 2 leaves. G and H: attached leaves. K and L: cut leaves. G and K: virus-spreading 20 hours after inoculation. H and L: virus-spreading 22 hours after inoculation. Numbers 1: unwounded leaves; numbers 2, 3, 4, and 5, in order, injuries inflicted 72, 48 and 24 hours before and simultaneously with inoculation.

hours after inoculation or even earlier. The experiments were performed in duplicate and each diagram in Fig. 5 represents an average of two leaves. The leaves represented by G and H of this figure were leaves in situ; those of K and L, cut leaves. Diagrams G and K represent the results obtained at 20 hours after inoculation; diagrams H and L the results at 22 hours. The numbers 1 to 5 in each series of diagrams refer, in order, to the following treatments: uninjured controls, wounded at the base 72, 48 and 24 hours before and simultaneously with inoculation. In the experiment with attached leaves, only the leaves wounded 24 hours before inoculation contained virus-material in the wounded strip 20 hours after inoculation (G4). Both this treatment and wounding simultaneously with inoculation (G5) caused a more extensive virus-spreading than that obtained in the control leaves (G1). Twenty-two hours after inoculation a greater virus-transportation had taken place: all of the wounded leaves contained virus in the injured base (H2, H3, H4 and H5) while the bases of the control leaves were still virus-free (H1). In comparison with the uninjured leaves only wounding of a leaf-base 24 hours before or simultaneously with the inoculation influenced the virus-spreading markedly. In all the leaves of diagrams H3 and H5, a virus-free region between two virus-containing strips was demonstrated. A zone was considered to be virus-free if the number of local lesions caused by the sap of the strip was less than three. If such a zone was found in the corresponding strips of the two leaves, a virus-free zone was denoted in the scheme. In a few cases only one of the corresponding strips was virus-free. Then the total number of local lesions had to be less than 10 before the two corresponding strips were denoted as virus-free.

In experiments with cut leaves (K and L) nearly the same results were obtained at 20 and 22 hours after inoculation. Wounding of the leaf-base 72 hours before inoculation had no influence on the rate of transportation of virus-material at either 20 or 22 hours after inoculation. The wounded area was not reached by the virus-material at 20 hours. Injuring 48 hours before inoculation exercised the same effect in cut leaves as in attached leaves. The wounded area was reached by the virus-material 22 hours after inoculation. Wounding 24 hours before inoculation had the greatest influence on the rate of transportation. Twenty hours after inoculation all wounded parts were invaded. Wounding simultaneously with the inoculation increased the rate of virus-extension also as compared with the control leaves. Intervening virus-free zones were found in all leaves shown in diagrams K4, L3 and L5, indicating that there is a tendency for the wounded leaf-base to attract virus-material.

From the experiments with inoculated and wounded leaves it can be concluded that the spreading of virus-material in a leaf is influenced by wounding of the leaf-surface only when the injuries are made within the 48 hours-period before the inoculation. To ascertain the moment at which the virus-material begins to leave the inoculated region, the following experiment was carried out.

In six uninjured leaves of which three were attached to the plant and three excised, the spreading of virus-material out of the inoculated middle zone to the other strips was ascertained 18 hours after inoculation. In all cases transportation to one of the adjacent strips had taken place, but the number of local lesions, on *Nicotiana glutinosa* leaves, caused by the sap pressed out of these strips was small: 10 at the most. Therefore it may be assumed that the transportation of virus-material out of the inoculated zone did not start much earlier than 18 hours after inoculation. When injuries are inflicted 48 or 24 hours before inoculation, the conditioning period of the wounded cells will have lasted 48 plus 18 or 24 plus 18 hours, i.e. 66 or 42 hours before transport of virus-material started out of the inoculated strip. It may be assumed that the cells in the wounded region are in a state of optimal activity at the moment that the transportation begins. Wounding of the leaves 24 hours before inoculation caused the greatest virus-extension. Probably the conditioned cells are in a high state of activity about 42 hours after wounding. The influence of wounding, inflicted simultaneously with the inoculation appeared to be smaller and could be explainable by the fact that the wounded cells require some time to become activated.

From the experiments shown in Fig. 3 it became clear that wounded cells in stems demonstrated their optimal activity between 24 and 48 hours after wounding. Results of the experiments with wounded leaves support this conclusion.

6. THE RATE OF VIRUS-TRANSPORTATION IN WOUNDED AND UNWOUNDED PLANTS IN WHICH THE MERISTEMS WERE INHIBITED IN THEIR ACTIVITY

In the experiments so far described, the influence of the stemtips and the axillary buds on the virus-transportation has been determined by removing these meristematic parts. The effect of the absence of the meristematic tissues, however, could not be stated as the wounds made by their removal were of great influence on the rate of virus-transportation. Only the action of the activated cells at the wound-surface could be investigated. We, therefore, tried to determine the influence of stemtips and axillary buds on the rate of virus-transportation by eliminating their action without wounding. This was done by treating the stemtip and the axillary buds with fusarex, 2, 3, 5, 6; -tetra-chloro-nitrobenzene.

Five, six weeks old, plants were powdered lightly with this substance simultaneously with the inoculation of the middle leaf with sap containing TMV. As controls, five plants were inoculated with TMV but not powdered with fusarex. An additional five plants were treated only with fusarex. Fusarex completely inhibited the growth of the plants for about ten days; the activity of the meristems seemed to be eliminated and the plants remained in the same stage of development, which had been reached at the moment of inoculation. Ninety-six

hours after inoculation all the leaves were cut, placed in water and after three days tested for the presence of virus with the method described. The rootsystems were also tested (Table IX).

TABLE IX

Spreading of virus 96 hours after inoculation of the middle leaf and treatment of the tips and the axillary buds of the plants with fusarex.

Treatment	Virus-containing leaves	Ratio [3])	Virus-containing rootsystems
— [1])	6/25 [2])	0.24 ± 0.08	1/5
fusarex	1/25	0.04 ± 0.08	0/5

[1]) Five plants per group.
[2]) and [3]) See note Table I.

Results showed that only one uninoculated leaf from the five fusarex-treated plants contained virus. In the plants treated only with TMV, the virus had penetrated into one or two leaves on each plant in all cases. The treatment with fusarex had obviously inhibited almost all virus-transportation in addition to the meristematic activity. Plants treated with fusarex only, started further development about ten days after the treatment. The new growth showed a slight winding and sometimes a yellowing. Fusarex had apparently exercised a phytotoxic action. In the preceding experiments it was shown that cells near a wound are in an active state and accelerate the virus-transportation in a plant. Since fusarex-treatment appeared to have an inhibiting action on the transport through a plant, it can be asked, what the combined effect of a wound and fusarex-treatment would be. It may be possible, that the effect of wounding surpasses that of fusarex in the plant.

Therefore, in the following experiment the epidermis of the stem was slightly damaged along the whole length at two opposite sides. The trial was carried out with six groups of three plants, six weeks old, treated as follows:

1) all axillary buds and stemtips were powdered with fusarex, 24 hours before inoculation of the middle leaf and wounding of the stem.

2) fusarex-treatment, wounding and inoculation took place simultaneously.

3) the buds and stemtips were powdered with fusarex 24 hours after inoculation and wounding.

4) no fusarex was used, the stem was wounded simultaneously with inoculation of the middle leaf.

5) fusarex-treatment and inoculation occurred simultaneously, no injuries were made.

6) fusarex-treatment and inoculation occurred simultaneously, stems were not wounded and growth of the plants was observed following recovery of fusarex-treatment.

D. H. BRANTS:

The influence of meristematic tissue and injuries on the transport of tobacco mosaic virus in Nicotiana tabacum L. cultivar. Samsun

Plate 1. A: tobacco leaves of one plant in test-tubes containing water 3 days after cutting (1/2 ×); B: tobacco plant from above (3/4 ×); C: stemtop of *Nicotiana tabacum* showing 3 young leaves (3 ×).

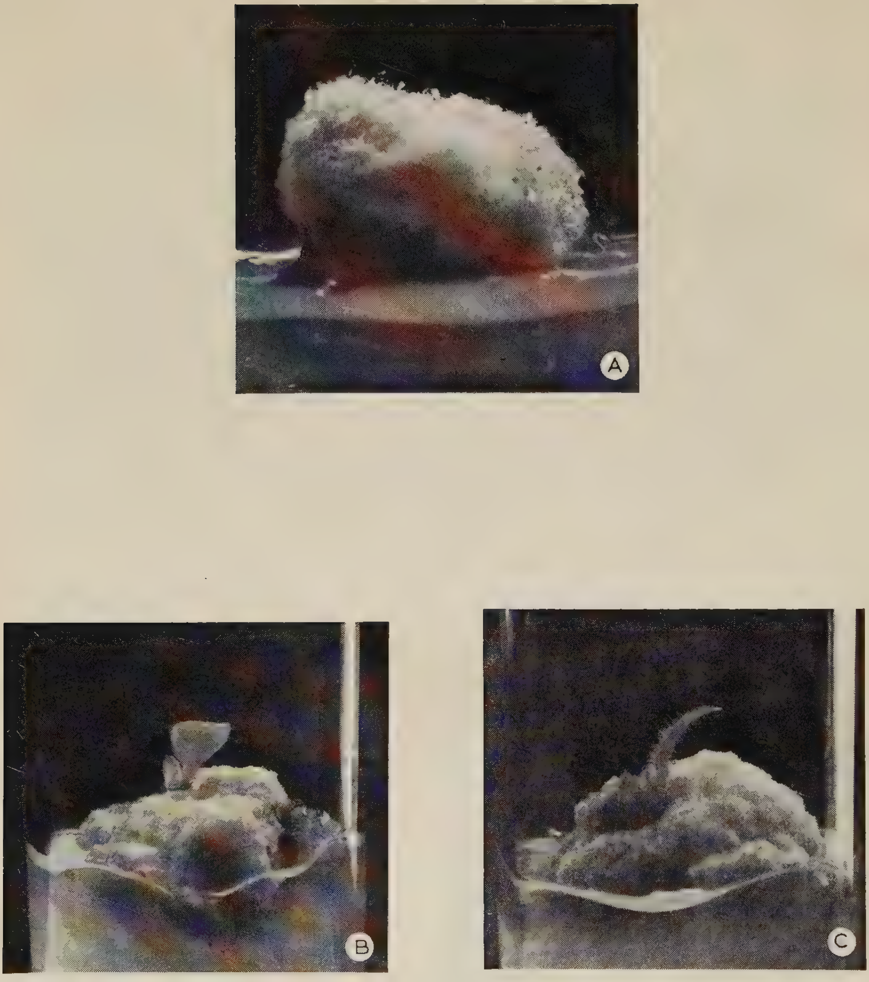

Plate 2. A: Healthy callus-tissue of *Nicotiana tabacum* (3 ×); B: Growing stemtip on callus-tissue 15 days after placing (2½ ×); C: Growing stemtip on callus-tissue 15 days after placing showing circumvallation of callus-tissue round the stembase (2½ ×).

The leaves and the rootsystems of the five groups were cut 96 hours after inoculation and then tested for the presence of virus as usual.

It became evident that virus-transportation had taken place only in the plants of the group not treated with fusarex. Five of the 17 tested leaves and two of the three root-systems were infected with virus. In all of the other groups none of the uninoculated leaves or root-systems contained virus. In eight of the twelve fusarex-treated plants, even the sap, pressed out of the inoculated leaves, did not produce local lesions on the leaves of *Nicotiana glutinosa*, even though the sap used for inoculation had shown a high concentration of virus when it was tested on Xanthi tobacco. The leaves of *Nicotiana glutinosa* used for the assay are known to have been in a good condition, since the sap pressed out of the leaves of the group not treated with fusarex, caused the normal number of local lesions.

It was shown that cells in a state of conditioning had not been able to reduce the effect of fusarex, since microscopically visible wound-reactions were the same for both the treated and untreated plants. It may be assumed from this experiment that fusarex prevents or inhibits virus-multiplication in the inoculated leaf to such a degree that subsequent transportation is impossible. The plants of the sixth group treated with fusarex and inoculated with TMV, showed virus-symptoms in the young leaves five weeks after the treatment. The inhibiting action of fusarex on virus-multiplication and transportation seemed to be only temporary. The purely negative results of this experiment made it impossible to determine whether there is a causal relationship between the absence of meristematic activity and the lack of virus-multiplication and transportation. It is possible that a small multiplication occurs, but that inhibition of food-transport causes not only stagnation in development of embryonic tissues but also cessation of virus-transportation if this is dependent upon the streaming of nutrients. The symptoms, shown by fusarex-treated plants, would indicate that damage of tissues in an active state of development would prevent not only virus-transportation, but also virus-multiplication in the leaves.

7. CALLUS-TISSUES

7.1. Introduction

A direct influence of meristematic tissue on the rate of virus-transportation in a tobacco plant has not been demonstrated by the preceding experiments.

Removal of meristematic tissues leads to injuries, which themselves influence the rate of virus-transportation. Inactivation of the meristems by treatment with fusarex seemed to inhibit virus-multiplication. Therefore, it was desirable to find another method to investigate the influence of meristematic tissue on the rate of virus-transportation. The use of callus-cultures seemed to be promising. If it were possible to introduce an active meristem or stemtip into an unwounded callus culture, its influence on the rate of virus-transportation within this

tissue might be determined. The fact that callus consists of homogeneous or nearly homogeneous tissue without vessels in which virus-transportation takes place only from cell to cell, would be another advantage. This may not have been the case in the leaves used in former experiments. Moreover, virus-transportation occurs only slowly in callus-tissue and an accelerating action of a meristem should be readily demonstrated. Considering these advantages callus cultures of tobacco were cultivated in vitro.

7.2. Literature

Much is known about the techniques of the cultivation of tissues in vitro. Practically each kind of tissue requires another medium (Gautheret, 1959). Several precautions are necessary in the cultivation of an homogeneous undifferentiated tissue, since certain substances added to the substratum are able to change the properties of the tissue. Auxins often cause differentiation, followed by stagnation in development and ultimate death of the cultures. Auxin added to the medium in concentration greater than 10^{-8} may induce the desorganized formation of vascular elements in the callus-tissue of tobacco. Virus-transportation over short distances will occur through these elements more rapidly than in homogeneous tissue. Since only undifferentiated homogeneous callus-tissue could be used for our purposes, callus-tissues of tobacco were cultivated on a medium containing auxins in concentrations of 10^{-8} (Morel, 1948). On this medium only a few or no vascular elements are formed in the tissue.

Camus (1949) studied intensively the influence of buds on the morphogenesis of callus-tissue of roots of *Scorzonera hispanica* L. Stemtops of these plants with a length of two to four cm, containing the top-meristem, the leaf-primordia and young leaves, grafted on a callus-tissue, inhibited the development and the growth of new buds out of the callus, induced vascular elements within the tissue and root-formation on its basal side. Camus could demonstrate a continuity between the vascular system originating from the grafted sprout and that of the newly formed roots. The histogenic influence exercised by grafted stemtops on the callus culture in vitro was comparable with that exercised by spontaneously developed sprouts in the callus-tissue. Such a spontaneous meristematic formation could be damaged mechanically in such a way that a deformed sprout, composed of irregularly shaped solid meristematic tissue without leaves, developed out of it. This sprout influenced the callus-tissue in the same way as a normal well-developed sprout with leaves. Therefore, Camus concluded that the histogenic influence is not exercised by the leaves of a sprout, but by the meristematic tissue.

To determine the rate of spreading of virus in tissue-cultures it is necessary to start with healthy callus which is inoculated with virus.

Different methods of inoculating callus-tissue with virus are mentioned in the literature. Not all of the methods have been successful. Kassanis, Tinsley & Quak (1958) obtained infected callus-cultures of tobacco by treating them with sap containing TMV and carborun-

dum-powder. BERGMAN & MELCHERS (1959) came to the conclusion that an infection succeeded only after wounding the tissue mechanically. However, WU, HILDEBRANDT & RIKER (1960) who infected callus cultures by shaking the cells in a nutrient solution containing virus, obtained infected callus-tissue without a carborundum-treatment.

The presence of virus in callus-tissue can be demonstrated either serologically or by application of the local lesion test with *Nicotiana glutinosa*. According to SCHRAMM & ENGLER (1958) the latter method appeared to be the more sensitive of the two, since a minimum concentration of 10^{-12} to 10^{-13} gram TMV/ml is detectable. The minimal infective dose appeared to be smallest in plants reacting with systemic symptoms, such as *Nicotiana tabacum* cultivar. Samsun. Using a considerable number of these latter plants, they found a 50 % infection with a dilution of 10^{-16} gram TMV/ml. This dose was equal to ten virus particles.

The concentration of virus in tissue cultures is always low. According to KASSANIS (1957) it is 30 to 40 times lower than in the leaves of a plant. HIRTH & SEGRETAIN (1956) detected quantities 40 to 50 times smaller than in a leaf, demonstrable with difficulty serologically. Inoculation of *Nicotiana tabacum* with the sap pressed out of the callus culture, the most sensitive method of demonstrating TMV in our experiments, proved to be technically impossible because of the great number of plants needed. Since the small amounts of TMV present in callus-tissue can be demonstrated also by the local lesion test, this method was chosen.

The growth of the callus-tissues appeared to be optimal at a temperature of 20° to 36° C and on media of pH 5.0 to 6.5; the virus-activity calculated per unit of tissue-weight was the highest at a temperature of 24° to 28° C and at a pH of 8.4, according to HILDEBRANDT, RIKER and WATERTOR (1954).

The virulence of the virus does not decline during culturing of the tissue (AUGIER DE MONTGREMIER, LIMASSET and MOREL, 1948).

Virus-multiplication per unit weight of tissue-culture was shown to be greatest during the third month after subculturing, when the growth of the culture declined (HIRTH and SEGRETAIN, 1956). An apparent antagonism seems to exist between virus- and cell-multiplication in the callus-tissue (WU, HILDEBRANDT and RIKER, 1960, HIRTH, 1960). According to KASSANIS (1957), however, the virus-concentration under conditioned circumstances remains constant and is not influenced by the growth of the callus.

Transport of TMV in callus-tissue of tobacco occurs at a rate of about one mm a week, according to KASSANIS, TINSLEY and QUAK (1958). This rate is about the same as that in leaf-parenchyma (KASSANIS, 1956).

Unequal growth in callus-tissue has been described by KLEIN (1957). He observed that cell-division takes place solely in definite regions of the superficial layers of the culture. It seems possible that the virus is spreading more quickly in these layers of the callus culture than in the centre. According to WU, HILDEBRANDT and RIKER (1960),

however, the virus-multiplication in these dividing cells should be smaller.

7.3. Material and methods

For the cultivation of callus-tissues in vitro, greenhouse-grown plants of *Nicotiana tabacum* L. cultivar. Samsun, with a length of 50 cm or more were used. Stem-parts of these plants were treated according to the method of Morel (1948) slightly modified. Though the surface of the stems were not contaminated with many micro-organisms, dis-infection was still necessary. This had to be done carefully, as the cambial zone is covered by only a thin layer of cortex. Leaves were stripped from the stem, the stem was cut into 30 cm sections; these sections were plunged into 96 % alcohol to remove all air from the surface. The stem-parts were placed in bleaching-liquor (80 g bleaching-powder per l water) with a maximum temperature of 28° C. After 25 minutes the parts were rinsed twice with sterile water and carefully peeled as far as the cambium, plunged into 96 % alcohol once more and placed in sterile water. The stem-parts were then divided into 1½ to 2 cm pieces. In general, only the basal end of the stem-piece, is able to form callus-tissue. As the contact of this end with the sub-stratum often inhibits growth it is necessary that this end be kept in the air above the substratum. Therefore the stem-pieces were placed on the medium with the apical end downward and the base upward.

The substratum, a KNOP-solution half diluted, contained per l distilled water:

$Ca(NO_3)_2 \cdot 4\,H_2O$	0.5 gram
KNO_3	0.125 gram
$MgSO_4 \cdot 7\,H_2O$	0.125 gram
KH_2PO_4	0.125 gram

3 % glucose,
0.7 or 0.8 % washed agar,
1 mg vitamin B_1,

1 cc HELLER-solution, containing per l distilled water:

$FeCl_3 \cdot 6\,H_2O$ . .	1 mg	$CuSO_4 \cdot 5\,H_2O$.	0.03 mg
$ZnSO_4 \cdot 7\,H_2O$ . .	1 mg	$AlCl_3$	0.03 mg
H_3BO_3	1 mg	$NiCl_2 \cdot 6\,H_2O$ . .	0.03 mg
$MnSO_4 \cdot 4\,H_2O$.	0.1 mg	KJ	0.01 mg

0.01 mg I.A.A. or N.A.A.

5 % coconut-milk, dependent on the condition of the culture.

The tubes with this medium were sterilized for 20 minutes at 108° C. After the stem-pieces were placed in the medium the stoppers were covered with plastic to prevent desiccation of the medium. The atmosphere in the tube was 100 % relative humidity. Unfortunately the percentage of sterile stem-pieces appeared to be low. Bacteria occurred inside the stem-pieces which sometimes developed a month after the pieces were placed into the tubes. The bacteria infected the medium. By adding five cc merthiolate-solution (containing 0.1 % merthiolate and 0.1 % mono-ethanol-amine) per liter medium better

results were obtained, because merthiolate inhibited the growth of bacteria and fungi without influencing the growth of callus-tissue.

At the basal side of the pieces, callus-tissue developed in four to five weeks after the beginning of culturing. This callus-tissue was removed with a sharp knife, under sterile conditions, and placed in another tube of medium of the same composition. The cut surface of the callus was brought into contact with the medium. By subculturing the tissue every four to six weeks, the callus could be kept alive. After this period the volume of the tissue had increased about three times so that the callus culture could be divided into two to four parts. In this way, it was possible to enlarge the number of cultures. After the first subculturing, the tissues were not yet accustomed to life in vitro, growth was often irregular, and the tissues remained in a labile condition. Some of the cultures developed well, others grew badly and died after some time. Results were increasingly better with each subculturing of the vigorously growing cultures, and finally the tissues became accustomed to the medium (Plate 2A). The callus cultures remained growing without differentiation of the tissue when the medium contained a concentration up to 10^{-8} of naphtyl-acetic acid (NAA) or indole-acetic acid (IAA). On one occasion, when the rate of growth declined somehow, for some unknown reason, coconut-milk was added to the medium, and caused recovery of the normal growth rate without inducing differentiation of the tissue.

If stem-pieces from plants infected with TMV and showing distinct virus-symptoms were used for the culture of callus-tissue, the resultant tissue was entirely penetrated by the virus and could not be used for experiments to determine the rate of virus-transportation. We attempted to inoculate healthy tissue-cultures with this material. By placing a slice of the virus-containing tissue in contact with a healthy tobacco callus culture, in more than half of the cases no infection took place. Therefore this method was abandoned.

A more satisfactory method was to inoculate vigorously growing healthy callus-tissue laterally with sterile sap containing TMV and carborundum. At different times after inoculation, slices about one mm thick were cut from the tissue parallel with the inoculated side of the callus. After each cutting the knife was disinfected. It is difficult to obtain slices exactly one mm thick and this irregularity was a source of error since the virus spreads only slowly through the callus-tissue. The sap was expressed by rubbing the slice on a rough glass-slide with the aid of a little glass stick and was tested for the presence of virus by the local lesion test on *Nicotiana glutinosa* leaves. The half-leaf method was used because it is more suitable for indicating presence of small quantities of virus. Although the cells of the cut slices could be kept alive for a short time on a nutrient medium in order to give the virus an opportunity to multiply this method was not satisfactory since the virus-concentration in the cut slices did not increase.

Local lesion tests on the slices disclosed that a sharp border always existed between those slices in which virus was present and those in which no virus could be detected. It is possible, of course, that a very

small amount of virus had penetrated the tissue but insufficient to cause local lesions. One of the difficulties in determining the rate of virus-transportation in callus-tissue is that the rate of growth of the callus is unequal at different sites and this inequality may cause variability in the rate of virus-multiplication. The rate of growth of the inoculated side of the culture usually was much smaller than that of the other sides. This probably resulted from wounds caused by contact with the carborundum-powder at inoculation.

Cultures of virus-infected tissue had the same appearance as healthy ones in both morphological characteristics and anatomical features. The difference between infected and uninfected tissues could be demonstrated only by inoculation of tobacco plants with the sap pressed out of the callus-tissues. This was also established by Morel (1948) and Hirth (1960).

The purpose of the experiments was to determine the influence of a sprout with an active meristematic tissue on the rate of virus-transportation in a homogeneous callus culture. For this purpose, well growing callus-tissue laterally infected with TMV was used.

Small sterile stemtips were placed loosely on the callus culture without damaging the tissue. The necessity for avoiding injury of the callus-tissues had become clear from preceding experiments in which wounding had shown a great influence on the rate of virus-transportation.

To obtain sterile stemtips, seeds of *Nicotiana tabacum* L. cultivar. Samsun were exposed to 0.1 % sublimate-alcohol for half a minute, rinsed with water for 30 minutes and placed in a petri-dish with two % water-agar. After ten days they had germinated and the small germs were placed individually on sterile medium of the same composition as that used for the callus cultures. The topmost stemtip containing the top-meristem, leaf-primordia and only one young leaf could be removed from the well developed plant and placed on a callus culture under sterile conditions (Plate 2B and C).

Top-meristems containing at most only one leaf-primordium, as used for so-called "meristem-cultures", were taken from older plants and placed on callus cultures. Only 10 % of these meristems grew out to stems with small leaves and this process sometimes required half a year. Stemtips placed on the callus-tissue on the other hand grew out to a normal sprout with leaves in more than half of the cases (30 of the 55). Therefore most of the experiments were carried out with stemtips although both exercised the same effect on virus-transportation.

7.4. Experiments on the influence of a stemtip on the rate of virus-transportation in callus-tissue

A great number of well-growing callus cultures [1]) were inoculated with TMV at one side. Sterile stemtips were placed on 25 of these tissues simultaneously with inoculation.

Development of the stemtips was visible after ten days. The callus-cells at the surface of the culture enclosed the base of the stemtip and by this circumvallation, the stemtip was held rather firmly on the tissue. Forty days later, no histological connection was found between the stemtip and the callus-tissue.

The rate of virus-transport in callus cultures with and without stemtip was determined at different times after inoculation. Determinations on cultures without stemtip were performed in triplicate at 5, 10, 14, 20, 25, 30 and 40 days after inoculation. Trials with cultures with stemtips were made in duplicate at 10, 20, 30 and 40 days after the inoculation. Callus cultures without stemtips were used as controls in these experiments and, in them, the virus appeared to move continuously through the tissue at a rate of about one mm a week; this agrees with the results of the experiments of Kassanis, Tinsley and Quak (1958) (Table Xa and Xb).

During the first 20 days there was no difference between the rate of virus-transportation in cultures with and without stemtips. After 30 days, however, in both cultures with stemtips, the virus was detectable in slices cut at four mm and six mm from the place of inoculation but not in that slice cut at five mm. Controls of the same age showed no virus beyond the slice cut at four mm. The stemtips also remained virus-free. About four weeks after the introduction of a stemtip, in one case even as soon as 18 days afterwards, rootlets, which penetrated into the medium, became visible at the base of the callus cultures. They appeared to be virus-free. Forty days after inoculation and introduction of a stemtip the virus appeared to have permeated rather suddenly, and to a greater extent, through these cultures than through the controls. Both the stemtip and the rootlets then contained virus.

When a meristem was placed on a callus culture, rootlets also were formed at the base, but it took a long time for this to occur. This delay probably was due to the slow development of the meristem. The cultures without a stemtip or meristem never developed roots.

More rapid virus-transportation in the superficial layers and a somewhat higher rate of spreading in the tissue as a whole would be expected to result from unequal callus-growth. However, virus-concentration in the whole tissue is so small that virus, present in only a few cells at the surface of the tissue, cannot be demonstrated. For that reason it may be possible that the rate of virus-transportation found, is too low. This error would, however, refer only to the superficial cell-layers.

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[1]) I am highly indebted to miss Dra. F. Quak for supplying callus cultures when our tissues were killed by gas.

TABLE X

A. Spreading of virus in callus-tissue of tobacco; each determination is the result of the corresponding slices of 3 cultures.

Number of days after a one-sided inoculation	Distance in mm from the place of inoculation									
	1 1)	2	3	4	5	6	7	8	9	
5	x	–	–	–	–					
10	x	x	–	–	–					
14	x	x	–	–	–	–				
20	x	x	x	–	–	–	–			
25	x	x	x	x	–	–	–			
30	x	x	x	x	–	–	–	–		
40	x	x	x	x	x	x 2)	–	–	–	

B. Spreading of virus in callus-tissue of tobacco on which a stemtip was introduced; each determination is the result of the corresponding slices of 2 cultures.

Number of days after a one-sided inoculation	Distance in mm from the place of inoculation									
	1 1	2	3	4 3)	5	6	7	8	9	10
10	x	x	–	–	–	–				
20	x	x	x	–	–	–	–			
30	x	x	x	x	–	x	–	–	–	
40	x	x	x	x	x	x	x	x	x	–

x virus-containing slices in all cultures.
– virus-free slices in all cultures.

1) Place of inoculation.
2) Virus present in 2 of the 3 tested slices.
3) Slices with a stemtip.

The sudden increase of the rate of transport in the tissues with a stemtip might be correlated with the appearance of roots. It can be asked which of the anatomical differences occurring in the tissues resulted in this increased rate of virus-transport.

7.5. ANATOMY OF CALLUS-TISSUES WITH AND WITHOUT AN INTRODUCED STEMTIP

Cultures were fixed in the fixative of Bouin (30 cc saturated picric acid-solution in water, 10 cc neutral 40 % formol and 2 cc concentrated acetic acid). After 24 hours fixation, the callus-tissues were washed in 80 % alcohol to which lithium-carbonate was added until discoloration occurred, upgraded through alcohol and benzene, and embedded in paraffin mp 58° C. Ten to twenty μ sections were made with the aid of a microtome. They were deparaffinized in xylene and graded down via alcohol and water to the staining-solutions. LANGERON'S (1942) double staining technique, using methylene-blue and ruthenium-red, was applied. Cellulose in the cell-walls was stained red, woody walls blue-violet.

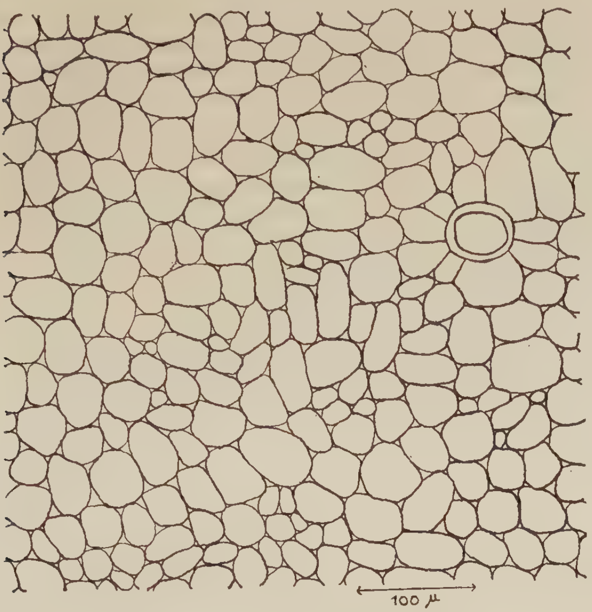

Fig. 6. Callus-tissue of *Nicotiana tabacum*. Undifferentiated tissue.

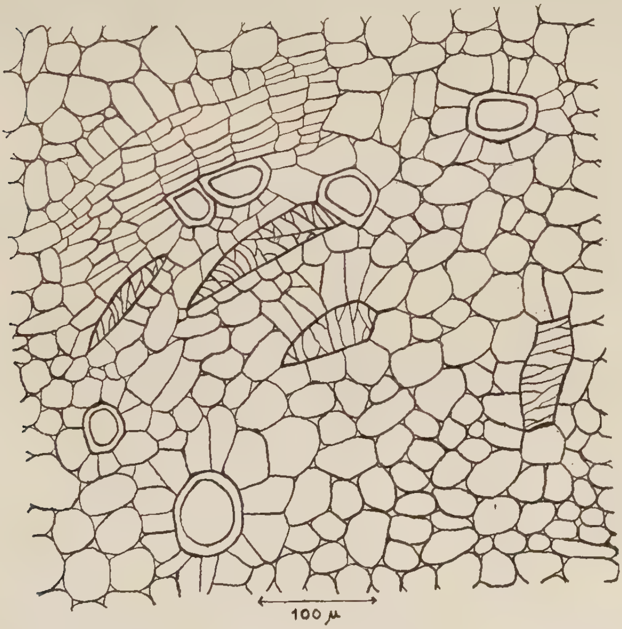

Fig. 7. Callus-tissue of *Nicotiana tabacum*. Occurrence of vascular elements in the tissue four weeks after placing of a stemtip on the callus-culture.

In general callus-tissue without a stemtip showed a homogeneous structure, but, on rare occasions a few vascular elements occurred (Fig. 6). In the tissue on which a stemtip had been placed, well defined changes were observed (Fig. 7). At four weeks vascular elements had developed throughout the whole tissue, and were particularly numerous near the base of the stemtip. In some cases vascular elements in the callus-tissue were oriented towards the vascular strands of the newly formed roots. The structure of these vascular strands in the callus-tissue appeared to be organized.

Introduction of a prepared meristem on the callus-tissue caused the same differentiation of the tissue as that of a stemtip, but was delayed. Although there still was no visible junction between the stemtip or meristem and the underlying callus-tissue, an organization of the tissue took place in both cases. The surface of the callus-tissue under the stemtip or meristem showed healthy cells without a trace of wounding, which were similar to the superficial cell-layers elsewhere. The cells of the cut surface of the stemtip had formed a continuous layer of dried cells and showed the same picture that has already been described for wound-surfaces in normal plants (p. 128). Since the healing-process took place within five days after introduction of the stemtip, it is impossible that wound-reactions resulting from cutting of the stemtip, were responsible for the accelerated virus-transportation which occurred three to four weeks later.

Four weeks after the introduction of a stemtip, transformation and organization of callus-tissue into vascular elements had occurred. It is known that virus-material is transported rapidly through vascular elements and these newly formed directive elements are no doubt responsible for the increased rate of transportation observed in these cultures.

The fact that the virus could not be demonstrated in some sections and that it was present in the adjacent ones indicates that virus-material passed through these sections without leaving any infectious material behind. This phenomenon is well known in stems (Samuel, 1934; Kunkel, 1939; Beemster, 1958) and has been found in our experiments with leaf-parts (Figs. 4 and 5).

The fact of organization makes it unlikely that a stemtip or a meristem, introduced into a callus-tissue, directly influences the rate of virus-transportation in the callus culture. An indirect accelerating influence on the rate of transport is caused by the differentiation induced in the callus-tissue. This is in accordance with the results of Camus (1949), who observed that histogenic changes occurred in callus-tissue of the roots of *Scorzonera* under the influence of a sprout or meristematic tissue.

Although there was no histological connection between the stemtip and the callus-tissue, under influence of this tip the callus began to function as a connection between the stemtip and the newly formed roots, i.e. as a stem with vascular elements. Transportation of material is such a potential plant could occur in a way comparable with that in a normal plant.

The rather sudden increase of the rate of virus-transportation in callus-tissue with a stemtip may be expected at the moment that the vascular elements are formed. The low rate of virus-transportation in undifferentiated callus-tissue can be explained by the absence of communication, with transport occurring only from cell to cell.

The view is widely held that viruses move from cell to cell of leaf-parenchyma through the protoplasmic strands (SHEFFIELD, 1936; SIEGEL and WILDMANN, 1960). As the rate of transportation in callus-tissue is about the same as that in leaf-parenchyma (KASSANIS, 1956) it may be expected that callus-tissue also contains protoplasmic strands (plasmodesm). It is however impossible to demonstrate them with the usual fixation- and staining-methods.

Electron photographs (KASSANIS, TINSLEY and QUAK, 1958) have shown that thin places occur in the cell-walls of callus-tissue. These places may play a rôle in the spreading of virus.

To demonstrate the occurrence of plasmodesm, if present, in the callus-tissue the method described by LAMBERTZ (1954) was used. One cm cubes of tissue were fixed in Gilson-solution for 22 hours (40 cc 30 % alcohol, 10 cc concentrated acetic acid, 5 cc 40 % formol and 1 cc 65 % HNO_3, saturated with sublimate), washed in 50 % alcohol, containing jodine. The tissue was cut into sections 25 μ thick with a microtome, the sections were held for five to ten minutes in a 20 % JKJ-solution, stained with sulphuric acid-pyoktannin-solution, rinsed in water, and placed in glycerine for observation under the microscope. In normal pith-tissue of the stem of tobacco treated in this way the plasmodesm were clearly distinguishable. In the cell-walls of the callus-tissue places were visible where the

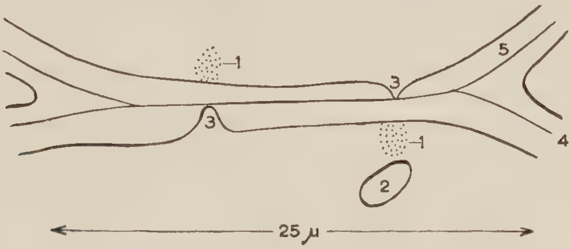

Fig. 8. Cell-walls of callus-tissue of *Nicotiana tabacum* after staining with sulphuric acid-pyoktannin. 1: granulation. 2: nucleus. 3: thin place. 4: middle lamella. 5: cell-wall.

secondary thickening layers were lacking or where they were scarcely present. Opposite these places, at the other side of the middle lamella in the neighbouring cells, a strong granulation in the cytoplasm occurred (Fig. 8). It is possible that at these thin places in the cell-wall an exchange of material takes place through the walls. No real plasmodesm could be demonstrated. The thin places are probably the same as those found by Kassanis, Tinsley and Quak.

8. DISCUSSION

The influence of meristematic tissue on virus-transportation in plants was studied in three different ways. One of the methods used in this respect by several authors was also applied here. The influence of removal of meristematic tissue on the rate of virus-transport was studied. If meristematic tissue attracts virus-material present in the plant, removal of the tissue would cause a change in the rate of transport. To estimate the rate of virus-transport in a plant it was determined in all our experiments at which moment still uninfectious virus-material transported from an inoculated leaf appeared in the uninoculated leaves. For this purpose all leaves of a plant were cut at a definite time after inoculation and kept seperately on water for three days (Plate 1). During this period virus-material, already present in the leaves at the moment of cutting, was allowed to multiply and to become infectious. Then its presence could be demonstrated.

Experiments showed that inoculation of a middle leaf caused the most rapid virus-transport (Table I, II and III). Removal of the stemtip did not influence the rate of virus-transportation out of the inoculated leaf (Fig. 1). Removal of the axillary buds or both axillary buds and stemtip caused an increased rate of virus-transport (Table 4). From these experiments it seemed as if in normal plants the presence of axillary buds exercises an inhibiting action on the rate of virus-transport. However, when the stem was slightly wounded along the whole length without removal of plant-parts, the same result was obtained (Table V and VI). Apparently it was the injury that accelerated virus-transportation in both cases. Therefore remarks mentioned in the literature, suggesting an attractive or a directing action of vegetation-points on virus-transport are doubtful in case these suggestions are based on experiments in which meristematic tissue is removed. At least during 72 hours after this treatment virus-material is attracted by the wounded tissue.

It is known that an injury is followed by wound-reactions, consisting in an increased respiration, an increased rate of streaming of the protoplasm in the cells and coating of the walls with suberin of cells bordering the wound. In some plants wounding may lead to the formation of a wound-meristem and wound-cork.

The reversion of mature cells to the juvenile state is ascribed to the action of wound-hormones which are produced only during a definite period after injuring. According to Braun (1952) the condition of the cells beneath the wound-surface in tomato plants is determining for the occurrence of a tumor in the presence of virulent crowngall bacteria. This "state of conditioning" occurs 24 to 96 hours after wounding with a maximum activity from 48 to 72 hours after a wound has been made.

The influence of cells in an activated condition at the wound-surface of tobacco plants was studied. Experiments were carried out on the effect of wounding at different times in relation to the moment of inoculation on the rate of virus-transport. Influence of the wounded

cells could be demonstrated from 0 to 72 hours after wounding with an optimum from 24 to 48 hours (Fig. 3, Table VII and VIII). The only microscopically visible reaction of tobacco tissue to wounding appeared to be a suberin-coating of cells. This process takes place in the first 48 hours after wounding (Fig. 2). Though after that time no reactions could be observed, physiological activity seems to occur until somewhat over 72 hours after wounding. From these experiments it became evident that cells of tobacco plants are activated after wounding and that these cells attract virus-material reaching the attraction sphere of the wound during the conditioning phase, lasting from the moment of wounding until somewhat over 72 hours afterwards. The duration of the conditioning phase agrees with that determined by Braun, although quite different material and methods were used.

The influence of wounding on the rate of virus-transport was also determined in leaves (Fig. 4). It was possible to check the moment at which virus-material reached a wounded leaf-strip after another part of the leaf was inoculated. Also in leaves, wounds seemed to accelerate the rate of virus-transport. Sometimes virus-material was transported so quickly in the direction of the wound that no material was left in zones between the inoculated and the wounded parts of a leaf. All effects of wounding of leaves supported the conclusions that virus-material appeared to be attracted by wounds during a period from 18 to 66 hours after injuring. Optimal attraction occurred 42 hours after wounding (Fig. 5). The results of the experiments with wounded leaves were in agreement with those obtained from the experiments with plants.

The results of all experiments suggested that wound-reactions influence the rate of virus-transport and it could be concluded that elimination of meristematic tissue by cutting was not a suitable method for studying the influence of this tissue on virus-transport.

The second method applied to investigate the influence of meristems on the rate of virus-transportation was based on elimination of meristem-activity without inflicting a wound. This purpose was reached by powdering the stemtip and the axillary buds of a tobacco plant with fusarex, a compound used for inhibiting the development of sprouts of potato tubers. After this treatment the plants remained in the original stage of development during ten days. When the middle leaf of such a plant was inoculated with TMV a very limited virus-multiplication took place but no virus-transport out of that leaf occurred (Table IX). Ten days after treatment the inhibiting action of fusarex was terminated and the plants started growing again. After five weeks, treated plants showed virus-symptoms. Many leaves were somewhat deformed or discolored showing that fusarex exercised a phytotoxic action. Probably not only the meristem-activity was inhibited by the fusarex-treatment, but also the metabolism of the plant was changed to such an extent that virus-multiplication was impossible and, consequently, nothing could be concluded about the influence of meristems on the rate of virus-transportation. Also in this way no decisive answer was obtained.

From the experiments it can be concluded that cells in an active state attract virus-material. This conclusion is not new since it is known that virus-symptoms first become visible in the young developing leaves, which seem to be suitable for virus-multiplication. It is also known that food-transportation occurs to young cells, as a food-stream will move from the place of food-production, the leaves, to places of food-consumption, such as young tissues or wounds and to places of food-storage. In general nutrients will move from "a source to a sink" (Crafts, 1951). According to Kursanov (1961) the movements of organic substances through the plant are apparently very complicated and the transport of organic materials over long distances is dependent not only on the metabolism of the conducting strands but also on the activities of the organs at either end. Both composition and direction of flow vary considerably for a variety of reasons. Young leaves that have not completed their growth continue for a long time to receive products from mature leaves. Even when they are able to photo-synthesize they do not yield their assimilates to other parts of the plant. These data will explain a great deal of the results of our experiments if it is assumed that virus-transport is correlated with food-transport (Bennett, 1956; Roberts, 1950). It would appear that not the primary meristematic tissue itself, such as vegetation-points, attract virus-material, but the active young tissues formed by the primary meristems, such as leaf-primordia, and mature cells transformed to the juvenile state by wound-hormones. To the actively growing tissues or cells in the "state of conditioning" nutrients and also virus will be transported as they will act as the "sink".

In order to answer the question of direct influence of meristematic tissue itself on the rate of virus-transport, a third series of experiments was performed with tobacco callus-tissue. It was possible to introduce a stemtip of two mm length, cut from a young seedling, or a meristem of about 200 μ, prepared from an older shoot, into the callus-tissue without wounding the culture (Plate 2). The use of callus-cultures made it possible to avoid a disadvantage of the experiments with leaves, in which virus-material is transported in two different ways, a quick transportation through the nerves and a slow transport by diffusion from cell to cell in the parenchyma. Tobacco callus cultures consist of homogeneous tissue in which virus-transport takes place very slowly from cell to cell at a rate of about one mm a week. The rate of virus-transport in a culture could be studied after lateral inoculation with TMV. A further advantage of using tobacco callus-tissue was that only one meristem or stemtip, introduced into the callus-tissue, could influence the rate of virus-transport in the tissue, which is impossible in a plant in possession of many axillary buds. Moreover no injuries had to be inflicted upon the callus-tissue by introduction of the meristematic tissue.

The first 20 days after lateral inoculation with TMV there was no difference in the rate of virus-transport in callus cultures with and without an introduced meristem. Thirty days after inoculation the rate of virus-transport was suddenly increased in the callus cultures

with meristematic tissue in comparison with the controls. Moreover, virus-free parts between two virus-containing regions were found, pointing to an attractive action of the developing meristematic tissue as occurred in wounded leaves (Table X). From these experiments it seemed as if a distinct influence of the introduced meristem on the rate of virus-transport was present. During the 30 days elapsed after introducing a stemtip, the callus cultures had formed rootlets. This was probably due to the influence of the introduced meristematic tissue, since root-formation has never been observed in callus cultures without such a treatment.

Microscopical observations of homogeneous callus-tissue without stemtip showed undifferentiated tissue (Fig. 6). Probably thin places in the cell-walls are responsible for the virus-transport from cell to cell since it has been impossible to demonstrate the presence of plasmodesmata thus far (Fig. 8).

The anatomical differentiations observed in the tobacco callus-tissue on which a stemtip or a meristem had been placed, consisted of the occurrence of vascular elements. The callus-tissue was no longer homogeneous (Fig. 7). No histological connection was found between the stemtip and the underlying callus-tissue. The histogenic influence of the tip must probably be ascribed to an hormonal effect. This is in agreement with the results of the experiments of Camus (1949), who showed that a graft separated from the tissue by a cellophane membrane still caused histogenic changes. It is probable that the formed vascular strands are responsible for the increased rate of virus-transportation in callus-tissue on which a stemtip was placed. The occurrence of virus-free zones between two virus-containing regions suggests that a rapid virus-spreading through the newly formed vascular elements had taken place. Here the meristem did not influence the rate of transport directly, but indirectly by inducing a differentiation of the tissue. The homogeneous callus-mass was changed into a potential plant with a stemtip, vascular tissue and roots.

It could not be demonstrated by all three series of experiments that primary meristematic tissue itself attracts virus-material. The directing action, mentioned in the literature, that has been attributed to top-meristems, apparently does not derive from the meristems themselves, but from the active young tissues formed by the primary meristematic tissues. Thus primary meristems may be considered to influence the rate of transport of virus-material indirectly. Stemtips, introduced on callus cultures, influenced the rate of virus-transport also indirectly by induction of vascular elements in the callus-tissue. Cells in the neighbourhood of a wound, returning to a juvenile state, may be considered as a zone of influence on the rate of virus-transportation during the "conditioning phase".

SUMMARY

1. The influence of meristematic tissue on virus-transportation in a plant was studied in *Nicotiana tabacum* L. cultivar. Samsun, inoculated with tobacco mosaic virus (TMV). The rate of transport of the virus in the plant was estimated by

determining the rapidity with which the virus appeared in uninoculated leaves. For this purpose the leaves of a plant were cut at different times after inoculation of a leaf and placed in water. Under this condition, virus-material present in the leaves at the moment of cutting was allowed to multiply for three days. After this period each leaf was pressed out and tested for the presence of virus by means of the local lesion test on leaves of *Nicotiana glutinosa* L.

2. From experiments in which either the lowest, the middle or the topmost leaf was inoculated, it became evident that inoculation of the middle leaf caused the most rapid virus-transportation.

3. The influence of meristematic tissue on virus-transportation was studied in different ways. One of the most obvious methods is to eliminate the meristematic tissue by removing it. In our experiments removal of the stemtip did not influence either the direction or the rate of virus-transportation out of the inoculated leaf. Removal of the axillary buds or both axillary buds and stemtip caused an increased rate of spreading of the virus.

4. When the stem was slightly wounded without removal of plant-parts the same result was obtained and appearently it was the injury that influenced virus-transportation.

5. Microscopical examination of the wound-reactions in *Nicotiana tabacum* revealed a suberin-coating of cells during the first 48 hours after wounding.

6. It became evident that wounding of the stem of a plant exercised an influence on the rate of virus-transport only when the wound was inflicted 24 hours before, simultaneously with or up to 48 hours after inoculation of a middle leaf. When the injury was made more than 24 hours before or more than 48 hours after inoculation, no influence was perceptible. Probably in the former case wound-reactions had already stopped and, in the latter, the virus-material had already extended too far.

From our experiments it became apparent that 48 hour-incubation of virus-material within a leaf is required before it comes out of the leaf. Then it may be concluded that wounded cells attract virus-material coming out of the inoculated leaf during a period lasting from the moment of wounding up to 24 + 48 i.e. 72 hours after that moment. If the virus-material reached the attraction-sphere of the wound 96 hours after injuring, no influence was perceptible. Optimal attraction by the wounded cells occurred from 24 to 48 hours after wounding.

7. The influence of wounding was also studied in leaves. One part of a leaf was inoculated, another part was wounded slightly at different times in relation to the moment of inoculation. The leaves were cut into sections 20, 22 or 24 hours after inoculation and these strips were kept on a fluid medium for four days in order to allow the virus-material present at the moment of cutting to multiply. Virus-material was transported quickly to the wounded region only when the injuries were made during a period from 48 hours before to simultaneously with the moment of inoculation.

As soon as 18 hours after inoculation virus-material begins to move out of the inoculated region of the leaf. So virus-material seemed to be attracted by wounds 48 + 18, 24 + 18 and 18 hours after wounding, i.e. during a period from 18 to 66 hours after injuring. The attraction was optimal 42 hours after wounding. All effects of wounding of leaves seemed to support the conclusion drawn from the results of experiments with wounded stems of plants.

8. According to Braun wound-activity occurs in a period from 24 to 96 hours with a maximum within 48 to 72 hours after wounding. This period is called the "conditioning phase". His results were obtained from experiments with *Agrobacterium tumefaciens* in wounded tomato plants. The duration of the "conditioning phase" found in these trials agrees with that found in our experiments, in which the effect of wound-activity on the rate of virus-transportation was determined.

9. Another method to investigate the influence of meristems on the rate of virus-transport was elimination of meristem-activity without inflicting a wound. The stemtips and the axillary buds of a tobacco plant were powdered with fusarex (2, 3, 5, 6-tetra-chloro-nitrobenzene), whereupon the plants remained in the original stage of development until ten days after treatment. The action of the meristems was inhibited. When the middle leaf of such a plant was inoculated with TMV a very limited virus-multiplication took place but no virus-transport out of that leaf occurred. Inhibition of meristem-activity and cell-growth diminished virus-multiplication

to a great extent and, consequently, the spread of virus through the plant. After ten days the inhibiting action of fusarex was terminated and the plants started growing again. After five weeks, treated plants showed virus-symptoms, many leaves were somewhat deformed or discolored. Probably not only the meristem-activity was inhibited by the fusarex-treatment but also the metabolism of the plant was changed and virus-multiplication seemed to be impossible.

10. A third series of experiments was performed with tobacco callus-tissue into which a prepared meristem or a stemtip was introduced without provoking injuries. The advantages of this method were:

a) virus-transport could be studied in homogeneous callus-tissue after lateral inoculation with TMV,
b) only one meristem could influence the rate of virus-transport in the callus, which is impossible in a plant in possession of many axillary buds,
c) no injuries were inflicted upon the callus-tissue by introduction of a meristem or a stemtip.

Though the meristems and stemtips developed into small stems with leaves, no histological connection was found between the callus-tissue and the "grafts".

Thirty days after "grafting" the callus-tissue had formed rootlets and the rate of virus-transport was suddenly increased in comparison with the controls without meristem or stemtip.

11. Anatomical observations demonstrated that the stemtip had induced a differentiation in the callus-tissue, consisting of formation of vascular elements. The callus-tissue was no longer homogeneous.

Microscopical observations of homogeneous tissue without stemtip revealed thin places in the cell-walls. Probably these places are responsible for the virus-transport from cell to cell in homogeneous tissue in which virus spreads slowly, about 1 mm a week. Apparently an introduced meristem did not influence the rate of transport in callus-tissue directly, but indirectly by inducing a differentiation of the tissue. The homogeneous callus-mass was changed into a potential plant with a stemtip, vascular tissue and roots allowing transport of material, including virus, in a way, comparable with that of a normal plant.

12. It could not be demonstrated by our experiments that primary meristematic tissue itself attracts virus-material. The directing action mentioned in the literature that has been attributed to topmeristems apparently does not derive from the meristems themselves but from the active young tissues formed by the primary meristematic tissues, such as leaf-primordia, which attract virus. Also mature cells reversed into a juvenile state by wounding exercise an attractive action on virus-material reaching the attraction-sphere of the wound during the "conditioning phase", i.e. during the first 72 hours after injuring.

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